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Sensitivity of UV-Irradiated Amoeba proteus to Hydrostatic Pressure[1]

ARTHUR M. ZIMMERMAN, HENRY I. HIRSHFIELD AND DOUGLAS MARSLAND

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Sol-gel transformations in a variety of cells have been under investigation for a number of years, but only in the last decade has it been possible to clarify the essential role of such reactions in relation to various cellular activities. In these studies hydrostatic pressure has been a useful tool in analyzing the significance of the sol-gel phenomenon (Marsland, '56). Similarly, studies concerning the effects of monochromatic light on living cells have afforded information as to the macromolecular composition of cells and the possible role of these components in cellular metabolism (Giese, '53).

Amoeba proteus lends itself well to studies on cellular activity. High hydrostatic pressures inhibit the amoeboid movement and cause pseudopodial collapse, resulting from a solation of the cortical gel structure (Landau, Zimmerman and Marsland, '54). Protoplasmic gels differ from other types of gels such as gelatin or oleate gels. Protoplasmic gelations conform to the type II system of Freundlich ('37) since they are endothermic ($-\Delta H$) in nature and proceed with a definite volume increase ($+\Delta V$) (cf. Marsland and Brown, '42). Therefore, the sol-gel equilibrium tends to be shifted toward the gel state by increasing temperature and toward the sol state by increasing pressure. Thus, both temperature and pressure have proved to be very useful parameters for analyzing how the various gel structures of the cell are related to physiological activity.

MATERIALS AND METHODS

Culture techniques. Vigorous cultures of *Amoeba proteus*, obtained from Dr. A. J. Dawson, were maintained by the method of Brandwein ('35), modified by the elimination of agar. The specimens were kept at 18°C, in the dark, at pH 6.9. Prior to use, 30–50 amoebae were thoroughly washed through several transfers of sterilized Brandwein solution and then subjected to treatment.

Pressure-temperature equipment. The pressure-temperature equipment was similar to that developed by Marsland ('50). The microscope-pressure chamber permits cells to be observed at magnifications up to 600 × while being subjected to pressures up to 16,000 lbs./in.^{2} Pressure is developed by the use of a modified hydraulic jack, which permits the pressure to be built up at the rate of 2000 lbs./in.^{2}/sec., with instantaneous decompression by means of a release valve. The temperature control chamber, which houses the pressure equipment as well as all glassware and solutions, is equipped with cooling and heating units, a circulating fan, and a bimetallic thermoregulator which permits the temperature to be set at any point between −5° and 60°C, with a maximum internal variation of ± 0.3°C.

UV-irradiation equipment. A Perkin-Elmer monochromater equipped with a 250 watt high-pressure mercury-argon lamp was employed as a source. The intensity of the monochromatic light, using a standard slit width of 1.0 mm is measured by a photomultiplier (1-P-28) previously calibrated against a standard UV

[1] Work supported in part by grants from National Science Foundation (G-6416) and (G-5963) and the National Cancer Institute (C-807), U.S.P.H.S.

lamp. The specimens were placed on a shallow fused quartz depression slide and irradiated from below.

Immediately after irradiation the amoebae were transferred to small lucite cups and placed within the pressure chamber at the desired temperature (20°C). Then, after 30 minutes of temperature equilibration, the cells were subjected to the pressure treatment.

RESULTS

Irradiation effects. The effects were most obvious at the lower wavelengths of 230, 265 and 280 mμ. With 265 and 280 mμ, at dosages of 6000 ergs/mm², there was generalized retraction of pseudopodia, accompanied by cytoplasmic blebbing, vacuolization and irregular cytoplasmic streaming. In most instances irradiations at 265 mμ produced the greatest effect on shape and activity. At 280 mμ these changes were not as marked. When the amoebae were irradiated at 230 mμ the damaging effects were drastic. About 60% of the specimens cytolyzed almost immediately after the treatment. Hence this wavelength was not employed in the subsequent pressure studies.

At the higher wavelengths of 302 and 365 mμ the amoebae retained a more normal appearance. They displayed extended pseudopodia and active cytoplasmic streaming, although they did show some surface blebbing and many had bulbous tips at the ends of the advancing pseudopodia.

Pressure effects. Since temperature (as well as pressure) has a decided influence on the form and movement of amoebae, the temperature was kept constant at 20°C, while the pressure required to produce pseudopodial collapse, rounding, and quiescence was being tested. Since it has been shown previously that these changes result from a pressure induced solation (loss of gel strength) in the plasmagel layer of the cytoplasm, it seemed logical to employ the same criteria in testing the UV effect on pseudopodial stability. Thus, the minimum pressure was determined which would produce complete rounding and quiescence in at least 70% of the specimens in each of the previously irradiated groups.

When control (non-irradiated) amoebae were subjected to 5000 lbs./in.² pressure, 77% of the cells became spherical within 20 minutes—which agrees essentially with the previous studies on *Amoeba proteus* (Landau, Zimmerman and Marsland, '54). However, amoebae which were irradiated prior to the pressure treatment showed a sensitivity which depended to some extent upon the specific wavelength of UV treatment. Irradiation at 280 mμ (6000 ergs/mm²) gave a very definitely increased sensitivity to pressure (see table 1). Thus at a pressure of 4000 lbs./in.², 71% of the specimens retracted their pseudopodia and became spherical, which represents a reduction of 1000 lbs./in.² from the control value.

In contrast to this result, other wavelengths (265, 302 and 365 mμ), used at the same dosage, gave very little effect. The form-stability of such irradiated specimens, when tested by pressure, proved to be essentially the same as that of the non-irradiated controls. This may be seen

TABLE 1

The effect of UV irradiation on the sensitivity of Amoeba proteus to the solating effects of hydrostatic pressure

Pressure	Percentage of fully rounded quiescent specimens				
	265 mμ	280 mμ	302 mμ	365 mμ	Controls
lbs/in.²					
5000	71 (179)	93 (82)	75 (68)	79 (87)	77 (541)
4500	58 (180)	76 (58)	49 (135)	40 (122)	56 (97)
4000	56 (147)	71 (222)	40 (101)	—	48 (162)
3500	—	48 (55)	—	—	—

In each experiment, the percentage of completely rounded specimens, without any persisting pseudopodia, was determined after a standardized compression period of 20 minutes. The figures in parentheses indicate total numbers of specimens observed in each case. Fractional percentages are omitted, i.e., percentages represent nearest whole numbers.

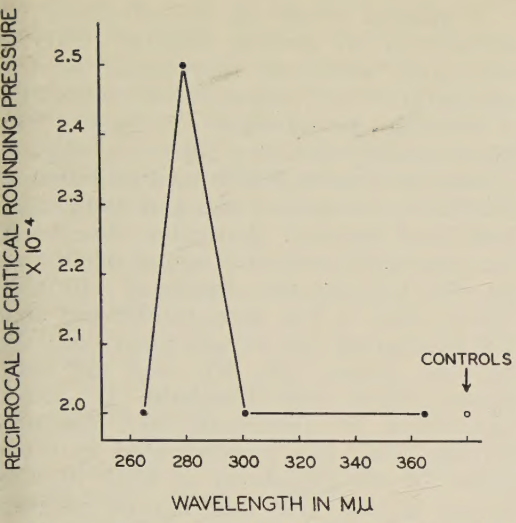

Fig. 1 The reciprocal of the critical rounding pressure is plotted as a function of wave length. Note that this graph presents a curve which may be regarded as a UV action spectra with reference to the pressure effects.

in table 1 and also in figure 1, which shows the reciprocal of the critical rounding pressure, plotted as a function of wavelength.

DISCUSSION

The results indicate that the plasmagel structure of the amoebae, upon which pseudopodial stability must depend, is most sensitive to damage by ultraviolet light in the 280 mμ wavelength region. If one were to study the effects of UV alone on shape and activity, without any concomitant pressure studies, one might infer that the 280 and 265 mμ wavelengths have very similar effects. Perhaps, in fact one might judge that irradiation at 265 mμ was more damaging. After UV irradiation at this wavelength, the pseudopodia were markedly retracted and there was extensive cytoplasmic vacuolization. However, when such amoebae were tested by pressure, it became evident that the gelational state was more closely related to that of the controls than to the amoebae irradiated at 280 mμ.

When the amoebae were irradiated at the higher wavelengths (302 and 365 mμ) or when the dosage was reduced (1500–3000 ergs/mm²), the pseudopodial stability, as tested by hydrostatic pressure, was comparable to that found in the control specimens. These relationships are summarized more clearly, perhaps, in figure 2, which graphically shows the percentage of amoebae capable of maintaining pseudopodia, at least partially, at a pressure of 5000 lbs./in.², applied subsequent to the various radiation treatments.

The studies of Hirshfield and collaborators ('58) clearly demonstrate nuclear dependence in regard to pseudopodial (cor-

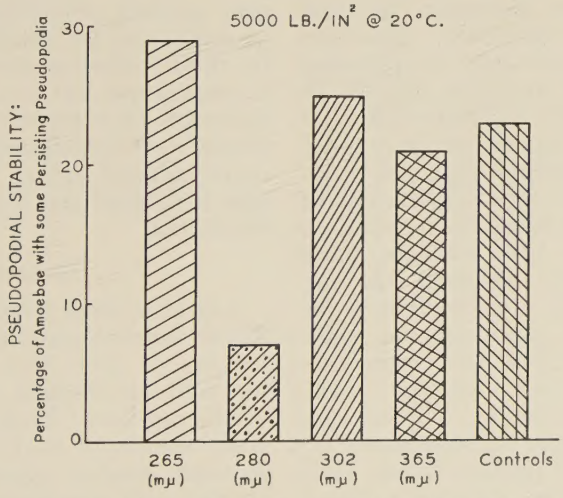

Fig. 2 The effects of monochromatic light on pseudopodial stability in *Amoeba proteus*. In each experiment the value illustrates the percentage of amoebae with definitely persisting pseudopodia after a compression of 20 minutes at 5000 lbs./in.² (20°C).

tical) stability. This might lead one to infer that UV-radiations at 265 mμ, which are so strongly absorbed by the nucleic acids, would diminish pseudopodial stability. However, the pressure experiments showed that there was little change in the pseudopodial stability, although pseudopodial activity was reduced very definitely, and distinctive changes occurred in the cytoplasm. On the other hand, irradiation in the protein absorbing region, 280 mμ, most probably affected the plasmagel layer directly and locally, weakening its intrinsic gel structure.

A particular sensitivity of protozoa to 280 mμ has also been demonstrated by Hirshfield and collaborators ('57). These UV - pressure studies on *Blepherisma* showed a maximum sensitivity to pressure lysis at 280 mμ, with dosage levels of 6000 and 12,000 ergs/mm². However, some nucleic acid components also may have been involved, since a distinct, though lesser, degree of sensitivity was displayed at 265 mμ.

The temperature-pressure studies of Marsland and associates have shown that amoebae display increased pseudopodial stability when immersed in weak solutions of adenosine triphosphate (ATP). Thus, it has been proposed that the plasmagel system of the amoebae can receive energy from the metabolism of high potential phosphate compounds in the cell (Zimmerman, Landau and Marsland, '58; and Zimmerman, '59). Moreover, anucleate amoebae are more sensitive to pressure solation than their nucleate halves or whole amoebae, which suggests that ATP utilization may be under nuclear influence (Hirshfield, Zimmerman and Marsland, '58). However, the ATP content of anucleate (half) amoebae is reported to be higher than in the nucleate parts (Skreb-Guilcher, '55), although the nucleate halves display a considerably decreased capacity to maintain the phosphorylation of ATP under anaerobic conditions (Brachet, '55). Brachet ('59) believes that this may be due to a decrease in diphosphopyrimidine nucleotide (DPN) synthesis, which is of nuclear origin. Thus with insufficient DPN, anaerobic glycolysis would be insufficient to maintain the phosphorylation of the ATP reserves.

A general review on the effects of UV irradiation on various cellular activities including amoeboid movement, nuclear regeneration and degeneration, represents a valuable contribution by Giese ('53). More recently Rinaldi ('59b) studied pinocytosis in amoeba following irradiation at 2537 A at dosages of 988 and 4944 ergs/mm², and reported distinctive changes in the contractile vacuolar output depending on the UV dosages employed (Rinaldi, '59a). Also it has been established that UV irradiation has effects upon both the nucleus (Giese, '45, '50) and the cytoplasm (Mazia and Hirshfield, '51; Skreb and Errera, '57; Hirshfield, '59). Recently Iverson ('58) has demonstrated that UV injury of the cytoplasm at 2537 A at a dosage of 1200 ergs/mm² can be reversed by replacement of the irradiated nucleus with a non-irradiated nucleus. However, non-irradiated cytoplasm was unable to reverse injury done to an irradiated nucleus and, in fact, non-irradiated cytoplasm seemed to be injured by the presence of an irradiated nucleus. Similar nuclear transfer studies by Ord and Danielli ('56) demonstrate that x-irradiation has an inhibiting effect on cell division (see also Giese, '53; Mazia and Hirshfield, '51).

Studies employing uptake of C^{14} phenylalanine as a measure of impairment of protein synthesis indicate that UV irradiation inhibits phenylalanine incorporation or exchange in amoebae (Iverson, '58). In these experiments it was found that incorporation of C^{14} phenylalanine was inhibited to a greater extent if the cytoplasm was irradiated and the nucleus non-irradiated rather than if the nucleus was irradiated and the cytoplasm was not irradiated.

SUMMARY

Amoebae were irradiated with a Perkin-Elmer monochromatic dosimeter at wavelengths of 230, 265, 280, 302 and 365 mμ and at dosages of 1500, 3000 and 6000 ergs/mm². Then the treated animals were compared with normal controls as to form, general appearance and capacity to maintain pseudopodia when subjected to the solational effects of high hydrostatic pressure.

Dosages at and below 3000 ergs/mm² produced no overt effects at any of the wavelengths and these irradiated specimens showed the same pressure-solation sensitivity as untreated controls. At 6000 ergs similar results were obtained at wavelengths 365 and 302 mμ, but with 280, 265, and 230 mμ treatments very definite effects were observed. The 280 and 265 mμ specimens displayed reduced pseudopodia and a vacuolated cytoplasm. However, only the 280 mμ group showed an increased sensitivity to pressure solation (endpoint at 4000 lbs./in.²) in contrast to the normal 5000 lbs./in.² The 230 mμ group, on the other hand, showed about 50% cytolysis and, therefore, were unsuitable for pressure testing.

The results suggest that the effects upon the plasmagel system of 280 and 265 mμ irradiations, although superficially similar, may be fundamentally different.

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Comparative Studies of Homotransplantation in Fishes[1]

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The notion that so-called lower or poikilothermic vertebrates are immunologically more primitive and hence less likely to respond vigorously to antigenic stimuli is still widely held (Cushing and Campbell, '57; Favour, '58). Although successful homotransplantation in anuran and urodele amphibian adults as well as larvae has been reported in a number of studies (cf. Hildemann and Haas, '59), failure to control the temperature and lack of adequate criteria for assessing homograft survival make the results questionable. A comprehensive study of the bullfrog by Hildemann and Haas ('59) showed that from larval stage 25 on this species regularly destroys skin homografts by a sensitive isoimmune response which is, however, temperature dependent. Nevertheless, similar investigations of various other species of amphibians need to be made before comparative generalizations can be asserted without qualification.

Among fishes, the homograft reaction has been most extensively studied in the goldfish (Hildemann, '56, '57, '58; Hildemann and Owen, '56). In this species the immune response to skin (scale) homografts was found to be closely temperature dependent. Fish maintained above 20°C responded in an exquisitely sensitive manner in terms of both outwardly visible inflammatory reactions and median survival times. Thus the median survival times of first-set homografts between unrelated goldfish ranged from 40.5 ± 1.9 days at 20°C to 8.3 ± 0.3 days at 21°C to 4.3 ± 0.1 days at 32°C. The higher the water temperature, the more rapid and conspicuous were the manifestations of homograft incompatibility. Kallman ('60) recently found that inter-strain fin homografts between two inbred strains of platyfish (*Xiphophorus maculatus*) derived from a common ancestral stock were rejected within 5–15 days, while F_1 to P_1 grafts survived 8–67 days. Although whole fin transplants pose technical difficulties leading to considerable degeneration even of autografts before regeneration takes place (Kallman and Gordon, '57), it is clear that acute rejection of homografts is usual in platyfish. Early work by Goodrich and Nichols ('33), Sauter ('34) and Nardi ('35) employing various fishes, revealed that homotransplants comprising scales, muscle, and ribs completely degenerated, while autotransplants succeeded. However, no controlled, quantitative studies were undertaken by these investigators. Unlike the seemingly contradictory results published on amphibians, rejection of normal tissue homografts in non-inbred fishes has been observed in all instances.

The main purpose of the present studies was to determine whether the highly developed capacity of the goldfish to give isoimmune responses is typical of teleost fishes in general. Several distinctive freshwater species native to both temperate and tropical habitats were employed. The findings will be considered in relation to the comparative immunology of tissue homotransplantation.

MATERIAL AND METHODS

The well-known goldfish (*Carassius auratus*), native to Asia, is found in all temperate parts of the world, as is the larger, more long-lived carp (*Cyprinus carpio*). In addition, three tropical species were studied: the black-spot barb (*Barbus filamentosus*), a Cyprinid fish like the above, which occurs in Burma, Ceylon and India; the blue acara (*Aequidens latifrons*),

[1] These investigations were supported by Research Grant C-4027 from the National Cancer Institute, U. S. Public Health Service.

a typical Cichlid fish found in Panama, Columbia, Venezuela and Trinidad; and the guppy (*Lebistes reticulatus*), a Poeciliid fish native to Venezuela, Trinidad and Barbados. Adults of the first four species were chosen because they have large, well-developed scales comprising epithelium and dermis with conspicuous chromatophores and a supporting scale plate. Such scales may be readily transplanted and observed *in vivo* under a stereomicroscope. Although skin and scales are often considered as separate entities by fishery biologists (Van Oosten, '57), "scales" or scale plates are dermal derivatives of the skin and constitute an integral part of the integument. The terms skin and scale will be used synonymously in reference to the whole, viable unit transplanted, while the calcified bony and fibrillary subunit will be called the scale plate in accord with earlier usage (Goodrich and Nichols, '33; Hildemann, '57).

Uniformly distributed, non-migratory chromatophores are especially suitable indicators of general graft viability. This has been shown by histological sections as well as by biological tests of survival of homografts at various stages of breakdown. The techniques of scale grafting and determination of graft survival end points previously described for the goldfish (Hildemann, '57) were applied in the present experiments.

Tricaine methanesulphonate at a concentration of 70 mg per liter of 0.7% saline provided sufficient anesthesia for grafting all species except the blue acara. However, it was inadequate at any safe dosage to fully anesthetize the blue acaras. With this species 70 mg tricaine plus 0.025 ml quinaldine per liter of 0.7% saline worked well synergically to provide safe, general anesthesia at 25°C. Fish were immersed in saline-anesthetic during scale grafting, but tap water-anesthetic was used for subsequent inspections. All experiments were done at 25 ± 0.5°C and fish were acclimatized to this temperature for at least one week prior to grafting. The animals were individually tagged through the caudal peduncle when received and were well-fed daily with commercial dried fish food or Gordon's formula (Gordon, '50).

RESULTS

In a pilot experiment with 4 adult, wild-type female guppies, reciprocal scale homografts and control autografts were made. Because the scale pockets are shallow and the scales small, it was difficult to fit the grafts properly. While the autografts were successful, the homografts were incompatible, as evidenced by graft melanophore breakdown and a cloudy epithelial hyperplasia in the contact zone with host tissue already apparent 48 hours after grafting. Because the scales are not directly invested with reflecting tissue, capillary circulation in the scales could not be distinguished with certainty from that in the underlying tissue. No vascular inflammatory reactions were observed. After 4 days, repopulation of homografts by host melanophores was already underway. Thus *in vivo* estimations of survival end points could not confidently be made and this species was considered unsuitable for comparative quantitative studies.

Reciprocal homografts were exchanged among 10 fish of each of the other species with a single control autograft on each individual. There was thus complete cross-grafting with each fish being both a donor and recipient with respect to all the others. Every autograft was permanently successful and showed no inflammatory reactions at any time. First-set homografts healed in as well as autografts and received a normal blood supply within three days as a result of inosculation of graft and host capillaries. However, all homografts were incompatible and were rejected after acute reactions. Second-set homografts broke down more rapidly than first-set homografts in all four species, thereby establishing the immunologic nature of the rejection.

The quantitative results are given in table 1. Data on skin homografts in bullfrog larvae are included for comparative purposes. The bullfrog study (Hildemann and Haas, '59) involved single skin homografts, 177 first-set and 43 second-set, exchanged between animals in pairs. Except for the goldfish, the total number of homografts scored for time of rejection differed more or less in the respective first- and second-sets. This was a consequence of host deaths or technical graft losses. Thus 16 out of 90 first-set homografts on the

TABLE 1

Comparative scale (skin) homograft survival times at 25°C

Species	Number of recipient animals	Total number of homografts scored		Median survival times ± standard error (days)[1]		Interval between first-set and second-set grafting days
		First-set	Second-set	First-set	Second-set	
Goldfish (*Carassius auratus*)	10	90	90	7.2±0.3(1.0)	4.7±0.2(0.7)	25
Carp (*Cyprinus carpio*)	10	88	82	7.5±0.3(1.5)	4.1±0.2(1.0)	22
Blue Acara (*Aequidens latifrons*)	10	74	85	7.2±0.4(1.8)	4.5±0.2(1.0)	25
Black-Spot Barb (*Barbus filamentosus*)	10	71	42	8.6±0.5(2.1)	6.0±0.3(1.1)	26
Bullfrog, standard stages 25–29[2] (*Rana catesbeiana*)	177	177	43	11.8±0.6(2.3) to 13.6±1.0(3.0)	5.1±0.3(1.0)	23–34

[1] Numbers in parentheses are the standard deviations of the MST's.
[2] Data taken from Hildemann and Haas ('59).

blue acaras were sloughed as a result of fighting while graft breakdown was in progress. Fortunately, the number of homografts observed in each set was still large enough to allow a meaningful quantitative analysis. The calculated median survival times (MST's) of the various sets of homografts and their 95% confidence limits provided the best available measure of the sensitivity of the immune response.

The most conspicuous and rapidly manifested inflammatory reactions were seen in the goldfish, where the MST's of first- and second-set grafts were 7.2 ± 0.3 days and 4.7 ± 0.2 days, respectively. Hemostasis, vasodilation and capillary hemorrhage (redness as seen grossly) in and around homografts was less pronounced in the carp, as was the superficial epithelial hyperplasia. Nevertheless, the two sets of homografts were rejected just as rapidly as in the goldfish, with MST's of 7.5 ± 0.3 days and 4.1 ± 0.2 days. Indeed, the second-set MST was the shortest of any computed at 25°C, although this result may be slightly misleading, since a number of grafts completely sloughed off by the 4th postoperative day may still have possessed surviving cells at the time. Only one second-set carp homograft showed a partial restoration of capillary circulation on the third day after grafting, but this had ceased by the following day. Cytotoxic reactions with respect to second-set grafts developed so rapidly that there was generally no restoration of graft circulation in either the goldfish or carp. This point is emphasized because the scales of both species are backed by a continuous layer of reflecting tissue which allows excellent *in vivo* observation of the entire vasculature of grafts.

While goldfish and carp scales in the gold phenotypes employed possess an abundance of large xanthophores as convenient indicators of viability, blue acara scales have uniformly distributed, corolla-type macromelanophores instead. These cells are non-migratory, unlike the dendritic or filamentous melanophores also present, which appear to have a phagocytic function in the skin since they concentrate in areas of trauma. Clusters of irregularly distributed blue and red pigment cells showing iridescence (iridophores) are also seen in the skin. The scales mostly lack reflecting tissue, making the capillary circulation difficult to observe. Although this Cichlid species is substantially different morphologically and physiologically from the Cyprinid species just described, the MST's of 7.2 ± 0.4 days and 4.5 ± 0.2 days for first- and second-set homografts are virtually identical with those of the carp and goldfish at 25°C. Minimal inflammatory reactions were observed during first-set rejection. While the hyperplastic reaction, involving a buildup of epithelial cells having a cloudy appearance, was more conspicuous

in most second-set grafts, redness resulting from extravasated blood was notably absent in both sets of acara homografts. One exceptional first-set homograft was still fully viable 14 days after grafting, but the comparable second-set graft was rejected after 6 days.

The least vigorous homograft rejections were observed in the black-spot barbs, though these fish were the most active of the species tested. The MST's of first- and second-set grafts were 8.6 ± 0.5 days and 6.0 ± 0.3 days. These values fall within the range of survival times characteristic of mammalian and avian skin homografts exchanged between animals of distant genetic relationship (see Billingham et al., '54). The overt inflammatory reactions accompanying first-set homograft rejection were much subdued in the black-spot barbs, but the hyperplastic response was nevertheless accentuated with second-set grafts as in the blue acara. While a normal capillary circulation was restored in autografts and first-set homografts within three days, such circulation was not observed in any second-set graft. Survival end-points were less readily determined in the barbs. Black, red, gold and blue pigment cells are found in the scale dermis and epidermis, but individual scales may lack one or two of these cell types depending on the body area. While ventral scales have a complete reflecting layer, scales above the lateral line are incompletely backed with reflecting tissue. Granulation of all pigment cells present in a graft was taken as the survival end-point. This was always associated in time with complete breakdown of reflecting tissue.

There have been only a few studies of the chemical composition of fish scale plates. Two scleroproteins, ichthylepidin and collagen, representing 41–84% of the scale plate in different teleost fishes have been identified. Their relative amounts appear to be nearly constant in various species, about 76% ichthylepidin and 24% collagen (Van Oosten, '57). Renewed inflammation of scale homografts associated with erosion and digestion of scleroprotein following soft tissue destruction was described in earlier work with goldfish. Moreover, an accelerated second-set reaction to scale plates was clearly evident (Hildemann, '57). With first-set homografts in the goldfish and carp at 25°C, the peak intensity of inflammation in host tissues investing the scale plates was reached 15 days after grafting, although plate digestion was already well underway at 11 days. Overt inflammation then slowly diminished, even though complete breakdown or sloughing of the scale plates required several more weeks. Scleroprotein erosion was apparent in the majority of second-set grafts after 5 days and maximal inflammation developed after only 7 or 8 days. On the whole, equally strong and rapid reactions were observed in the goldfish and carp at this stage.

The reactions against scale plate scleroproteins in the blue acara and black-spot barb were much less pronounced, being characterized by renewed epithelial hyperplasia without vascular damage to surrounding host tissue. Obvious scale plate erosion was evident in most first-set grafts on the barbs at 19 days, but only minimal disintegration occurred in acara grafts even after 25 days. Some destruction of second-set scale plates was first apparent in both species at 12 days after grafting, however. Thus scleroprotein in all four species must be regarded as isoantigenic and susceptible to dissolution *in vivo*.

DISCUSSION

It is apparent that the capacity to distinguish self from not-self is as highly developed in teleost fishes and anuran amphibia as in mice and men. With the likely assumption that temperatures around 25°C are physiologically normal for the poikilothermic species studied, one is tempted to conclude that the isoimmune response of fishes is even more sensitive and vigorous than that of higher vertebrates. The MST's of first-set homografts in the black-spot barb and bullfrog fell within the range of 8.5 to 12 days characteristic of mammals in general, though the barbs reacted at the most rapid extreme of the range. The goldfish, carp and blue acara, on the other hand, destroyed skin homografts more promptly than any other species on record. Moreover, the goldfish is also known to be capable of rapid production of humoral isoantibodies in high titers at comparable temperatures (Hildemann, '58).

Another manifestation of a vigorous omograft reaction in the fishes studied as the appearance of visible inflammaon in the contact area of graft and host ssues within three days after grafting. he most regular feature was a cloudy ithelial hyperplasia, while redness reulting from vascular damage was proment only in the goldfish and carp. No ch reactions were associated with the establishment of control autografts. rom a comparative standpoint, the severy of homograft inflammation observed as as follows: goldfish > carp >> blackot barb ≧ blue acara. While inflammaon was generally more conspicuous in cond-set grafts in all species, the intenty noted with respect to individual homorafts was not reflected in their survival mes. Comparable inflammatory reacons have been observed in amphibian nd mammalian skin homografts (Hildeann and Haas, '59; Converse and Rapaort, '56), though less clearly because of e opaqueness of the skin and absence reflecting tissue.

The technic of direct stereomicroscopic bservation *in vivo* revealed that the resration of capillary circulation in first-set in homografts was achieved by inoscution of graft and host vessels in all the ecies of fishes studied. This definitive rculation, often complete within 48–72 ours after grafting, ceased within a few ays as graft breakdown proceeded and pture of capillary walls with extravasaon of blood ensued. In fish, progressive growth of host vessels took place only ter the soft donor tissue overlying the ale plate had been replaced by host ssue. This heretofore controversial findg was established in the bullfrog as well. ecently, Rolle et al., ('59) in a detailed udy of vascular changes in mouse skin omografts concluded that circulation was a old graft vessels and not by way of w vessels formed after transplantation. s in the present study, they found that e time interval between grafting and e appearance of complete blood flow as too short to allow for formation of w vessels.

The steadily increasing body of evidence inting to the universality of skin homoaft rejection in adult vertebrates gains ditional support from the present findings with fishes. At present, Syrian hamsters constitute the only known exception in that skin homografts in various noninbred stocks show prolonged survival (Billingham and Hildemann, '58), but even in this species chronic rejection is the usual result (Adams et al., '56; Hildemann and Walford, '60). Indeed, the "uniqueness of the individual," in the apt phraseology of Medawar ('57) emerges as a fundamental biological concept. This uniqueness manifested by tissue or cellular incompatibility indirectly reveals the exceedingly complex genetic structure of vertebrate populations in general. There can be little doubt that such inborn diversity makes for versatility in evolution. Of more immediate significance is the probability that the great precision of the immune response is necessary to enable the individual to cope with a multitude of pathogens and foreign substances.

SUMMARY

1. Tissue transplantation reactions were studied in five species of fresh-water fishes native to both temperate and tropical habitats. Whole scale transplants in fishes have a number of advantages over skin grafts in birds and mammals for quantitative studies. Scale transplants require no suturing or bandages. Their relative transparency and the presence of reflecting tissue facilitate *in vivo* observations under the stereomicroscope. Large, nonmigratory chromatophores provide especially suitable indicators of general graft viability. Multiple grafts may be exchanged with ease and the degree of inflammation associated with homograft breakdown may be nicely scored. Moreover, the tempo of the homograft reaction may be varied according to the water temperature.

2. While control autografts were permanently successful, acute rejection of scale homografts occurred in all the species of fish studied at 25°C. Accelerated destruction of second-set homografts revealed the immunological basis of the rejections. The median survival times of first-set homografts were nearly identical in the goldfish, carp, and blue acara, ranging from 7.2 ± 0.3 days to 7.5 ± 0.3 days. Similarly, the MST's of second-set grafts

were 4.1 ± 0.2 days to 4.7 ± 0.2 days. These very vigorous homograft reactions demonstrated an exquisitely sensitive and highly developed capacity to give isoimmune responses. The MST's of first- and second-set grafts in black-spot barbs were 8.6 ± 0.5 days and 6.0 ± 0.3 days. These values fall within the range of survival times characteristic of mammalian and avian skin homografts exchanged between animals of distant genetic relationship. The guppy, a Poeciliid fish, also rejected scale homografts, but proved to be unsuitable for comparative quantitative studies.

3. The restoration of capillary blood flow in first-set homografts was achieved in all instances through inosculation of the original graft and host vessels within 72 hours after transplantation. This definitive circulation persisted for only a few days before hemostasis and graft breakdown ensued. Blood flow was rarely seen in second-set grafts and then only a partial, sluggish circulation of short duration was observed.

4. Overt inflammation associated with homograft reactions was characterized by a cloudy epithelial hyperplasia in the contact zone of graft and host tissues in all the species studied. Vascular damage with extravasation of blood was a conspicuous feature only in goldfish and carp homografts. The intensity of inflammatory reactions was generally accentuated in second-set homografts. Moreover, such inflammation appeared earlier and developed more rapidly in second-set grafts.

5. In the 4 species studied in detail, scleroprotein constituting nearly all the organic matter of scale plates elicited late inflammatory reactions associated with gradual erosion and digestion of homograft scale plates. This breakdown occurred following the destruction of soft donor tissue. Accelerated reactions to the scale plates of second-set grafts were also consistently observed. Thus such scleroprotein must be regarded as isoantigenic and susceptible to dissolution *in vivo*.

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ncorporation of Radioactive Sulfur in Hair

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During the course of a study on the ietabolism of sulfur amino acids in sur- ical wounds, it was noted that S^{35}-labeled mino acids not only appeared in the ound and the skin but were accumulated the hair as well. It has been shown that here is a marked change in the metab- lism of sulfur during the healing of ounds and that the sulfur amino acids ave an important role in the formation of eplacement protein in the wound (Wil- amson, '56; Williamson and Fromm, '55). he principal protein of human hair, kera- n, has been shown to contain approxi- ately 18% cystine and less than one er cent methionine (Lang and Lucas, 2). There have been several reports hich have indicated that S^{35}-labeled com- ounds are incorporated into the keratin f feathers and hair of laboratory animals Astudillo et al., '57; Tarver and Schmidt, 9, '42; DuVigneaud et al., '44; Edwards, 4). Not only have methionine and cys- ine been shown to become part of the hair rotein, but also S^{35}-labeled sulfate has een shown to be converted to cystine and hen incorporated (Astudillo et al., '57; lock and Stekol, '50; Block et al., '51). he results of the following experiments how that the sulfur from methionine or ystine, but not from sulfate, is taken up y human hair.

EXPERIMENTAL

The following experiments were carried ut on male surgical patients at Cook ounty Hospital. These men served as sub- ects in the study of sulfur metabolism in ealing wounds. Each subject was given pproximately 1 mc of S^{35} orally in the orm of either cystine, methionine, or sul- ate. Radioactivity was measured on the urgical incision and at other sites on the kin of the subject at various intervals fol- owing surgery. The measurements were made with a Geiger-Müller tube which was attached to a brass plate taped to the skin. An opening in the plate allowed measurement of the radioactivity in a standard exposed area while screening out radiation from the surrounding areas. The details of the measuring devices and technique of measuring radioactivity on the surface of the skin have been described elsewhere (Williamson and Haley, '60).

A considerable time after the administration of the cystine-S^{35}, it was found that some skin areas exhibited a relatively high radioactivity while at other sites it was quite low. This anomalous situation could be explained only by the fact that hair growth was greater in areas of relatively high radioactivity as compared to areas with low radioactivity. Following these findings, routine measurements were made on the scalp hair of experimental subjects after the administration of one of the S^{35}-labeled compounds. The data obtained are summarized in the following tables.

In table 1 is shown the relative levels of radioactivity in skin, wound, and hair after the administration of cystine-S^{35}. It will be noted that the S^{35} in the wound very rapidly rises to a high value and then gradually returns to the level observed in the skin. The level of radioactivity in the hair eventually becomes very much greater than that found in either the wound or the skin. However, the amount of radioactivity in the hair does not begin to increase until an appreciable time after the administration of the cystine-S^{35}. Much the same situation appears to hold after the administration of methionine-S^{35} (table 2). It should be noted, however, that the levels of radioactivity in the hair after the administration of methionine do not become as great as after the administration of cystine-S^{35}. When the

TABLE 1

Relative uptake of S^{35} after administration cystine-S^{35}

Subject no.	Days after administration	Counts/min./100 mm² *		
		Skin	Wound	Hair
1	4	81	398	—
	20	121	414	—
	82	48	65	4235
2	3	78	323	—
	32	122	103	804
	45	108	68	1345
3	3	140	405	—
	33	107	263	131
	39	104	225	508

* Corrected for background and decay.

TABLE 2

Relative uptake of S^{35} after administration methionine-S^{35}

Subject no.	Days after administration	Counts/min./100 mm² *		
		Skin	Wound	Hair
4	3	73	498	—
	13	110	758	10
	26	115	248	289
	55	73	103	790
5	5	43	255	—
	27	57	213	50
	41	48	158	64
	194	0	0	403

* Corrected for background and decay.

TABLE 3

Relative uptake of S^{35} after administration sulfate-S^{35}

Subject no.	Days after administration	Background	Counts/min./100 mm²		
			Skin	Wound	Hair
6	3	27	27	30	—
	10	24	30	28	—
	41	31	29	30	33
7[1]	44	34	32	—	38
	74	23		—	21
8[1]	44	34	35	—	36
	74	23		—	23

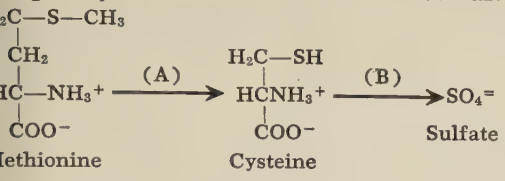
[1] Nonsurgical subjects.

source of S^{35} was sodium sulfate the level of radioactivity in the wound was only slightly higher than that observed in the skin; the skin was found to contain only an extremely small amount of radioactivity, and this disappeared within a few days (Williamson and Haley, '60). There appears to be no deposition of sulfate-S^{35} in the skin of either surgical or nonsurgical patients (table 3). At no time did there appear to be any accumulation of S^{35} in the hair, even after comparatively long periods of time.

DISCUSSION

It has been shown that the metabolism of sulfur is essentially an irreversible process following along the path indicated

elow, the fate of the carbon atoms being ompletely distinct from that of the sulfur.



lthough the over-all reactions represented y (A) are irreversible, there appears to e some evidence to indicate that the re- ctions represented by (B) may be rever- ible to some extent. This has been shown y the isolation of S^{35}-labeled cystine from he keratins of animals given S^{35}-labeled ulfate (Astudillo et al., '57). The sulfate on has also been shown to be utilized by he regenerating wound tissue of guinea igs (Kodicek and Loewi, '55). In this ase it was presumed that the sulfate was ncorporated into the mucopolysaccharides f the regenerating connective tissue.

A different situation appears to pertain o the metabolism of sulfur in humans Williamson and Haley, '60). Although nethionine and cystine appear to be in- orporated into the newly regenerating round tissue as observed in laboratory ani- nals (Williamson, '56), man does not eem to have the capacity to utilize the ulfur of the sulfate ion for incorporation nto either cystine or mucopolysaccharides. his is demonstrated by the fact that no ^{35}-labeled compounds appear in the regen- rating wound tissue of humans after the dministration of labeled sodium sulfate Williamson and Haley, '60). This fact further supported by the evidence pre- ented in this report, where no S^{35}-labeled ompounds appear in the hair of the sub- cts given labeled sulfate, in contradis- nction to the effect observed on the ad- ninistration of S^{35}-labeled cystine or meth- onine. In this latter situation, at least ome labeled sulfur should have appeared n the hair protein if there were any con- ersion of the sulfate to cystine. There ex- ts the possibility that the intestinal flora f man does not carry out the reductions hich convert sulfate to cystine. This ser- s of reactions is known to be brought bout by the enzymes of the bacteria found n several species of animals (Block and tekol, '50; Block et al., '51). In tissue ulture of human cells it has been shown that sulfate sulfur is not used in synthesis of cystine by the cells (Eagle, '58).

SUMMARY

The hair of human subjects given radiosulfur-labeled cystine and methionine accumulates much more radioactivity than does the skin. The high levels of radiosulfur do not appear in the hair until approximately 4 to 6 weeks after administration of the labeled compound. Although labeled cystine, methionine, and sulfate are converted to hair keratins in experimental animals, only the first two compounds are utilized in man.

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The Resting Potential and Intracellular Potassium of Skeletal Muscle in Frogs[1]

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It has been proposed that the membrane potential is determined by the ionic concentration across the membrane and the membrane permeability to the ions (Bernstein, '02; Hodgkin, '51). Consequently, the membrane potential has been expressed by Goldman's equation (Hodgkin and Katz, '49). If the relatively high membrane permeability to potassium ions is considered, Nernst's equation can be applied to the resting potential. In fact, the experimental evidence that there is a linear relationship between the value of resting potential and logarithm of extrenal potassium concentration has been known for a long time in frog sartorius muscles (Ling and Gerard, '49; Jenerick, '53; Harris and Martins-Ferreira, '55; Adrian, '56). Desmedt ('53) has reported the effect of decreased internal potassium on the resting potential by soaking preparations in low potassium Ringer to replace internal potassium with sodium, experiments which yielded results in good agreement with the ionic theory. Adrian ('56) has also studied the effect of alteration of intracellular potassium concentration and concluded that the membrane is not independent of the internal concentration at the physiological external concentration of potassium.

On the other hand, Tobias ('50) demonstrated that frog sartorius muscles soaked in distilled water maintained an average resting potential (39% of normal) despite the almost complete loss of intracellular potassium. Grundfest et al. ('54) and Falk and Gerard ('54) have brought about the alteration of the internal ionic concentration by means of micro-injection technique, and have found that alteration of internal potassium or chloride concentration does not cause the change in resting potential expected on the basis of the ionic theory. Recently, Shaw and his collaborators ('56) have reported that Nernst's (or Goldman's) equation is unable to account for the potential observed in individual skeletal muscles, stressing the necessity to offer an alternative hypothesis which accounts for the bioelectric potential. Stephenson ('57) and Stämpfli ('59) have also arrived to similar conclusions.

In the present experiment, the intracellular potassium content of isolated frog sartorius muscles was greatly altered by soaking in a hypotonic pure sucrose solution for various periods and the relationship between internal potassium and resting potential studied. The present study demonstrates that there is no correlation between these two factors and that muscles exhibit normal activity in spite of the marked decrease of internal potassium.

MATERIAL AND METHODS

Experiments were done between April to September, 1959 on frog (*Rana pipiens*). Sartorius muscles were carefully dissected out with all insertions intact.

The normal Ringer's solution had the following composition: NaCl, 112 mM; $CaCl_2$, 1.8 mM; KCl, 2 mM; $NaHCO_3$, 2.4 mM; pH 6.5. Some experiments were done in choline-Ringer in which 112 mM NaCl was replaced by 112 mM choline chloride. A 224 mM sucrose solution was made with ion-free distilled water and stored at 4°C. The hypotonic sucrose solution (112 mM) used throughout this experiment was made immediately before use by diluting 224 mM sucrose solution twofold with ion-free distilled water. The 224 mM sucrose solution was not kept for more than one week.

Isolated muscles were soaked in 112 mM sucrose solution (20 cm^3) for various

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periods at different temperatures; solutions were not renewed during the whole course of immersion. After a given period of immersion, muscles were transferred into Ringer's solution for electrical recording.

Glass capillary microelectrodes filled with 3 M KCl were used to measure the resting potentials of muscle fibers. After preparations were immersed in Ringer's solution for three minutes, the resting potentials of 10 fibers, chosen at random, were measured within the following 2–3 minutes. In some muscles the action potential (in Ringer), as well as the electrotonic potential for testing the rectifying property of the membrane (in choline), was recorded by means of two microelectrodes inserted close together into a single fiber. The preparations were used for potassium estimation immediately after potential measurements were taken.

As a routine, the wet weight of the muscle was measured before (initial wet weight = i.w.w.) and after (final wet weight = f.w.w.) the soaking procedure. The dry weight obtained from 10 muscles soaked in hypotonic sucrose solution for 30 hours at 10°C was 18.5–21.9% (average 20.4%) of i.w.w. On the other hand, the dry weight obtained from 10 muscles soaked in Ringer's solution for one hour at 10°C showed an average of 21.5% of i.w.w. Thus, it seemed that no serious error would be caused in the estimation of potassium by assuming that the dry weight of muscle did not change appreciably during soaking. In order to standarize the calculation, the dry weight was taken as 21% of i.w.w., regardless of the period of the following soaking procedure. The final total water weight was, therefore, figured as follows:

$$\text{Total water weight} = \text{f.w.w.} - 0.21 \times \text{i.w.w.}$$

The ratio of the extracellular space was not measured experimentally. It was assumed that the ratio of the extracellular space was not altered markedly after the soaking procedure. By taking the extracellular space of sartorius muscles as 12.5% (Boyles et al., '41; Desmedt, '53), the final intracellular water weight was expressed as follows:

$$\text{Intracellular water weight} = 0.875 \times \text{total water weight}$$

After muscles were reduced to ash the potassium was measured by using the Beckman's flame-spectrophotometer. Intracellular potassium was calculated by subtracting the interfiber potassium content from the total potassium content of a muscle (Boyles et al., '41). The amount of intracellular potassium is thus expressed in millimoles per 1000 cm^3 intrafiber water weight (mM/ifw).

RESULTS

When sartorius muscles were soaked in hypotonic pure sucrose (112 mM) solution, they gained water and increased wet weight. The value of the resting potential of muscle fibers, however, was well maintained over 100 mv. In fact, the resting potential was better maintained for an extended time in this solution than in any other potassium-free solution, such as potassium-free Ringer or saline, potassium-free choline or sucrose Ringer (containing 1.8 mM $CaCl_2$), and hypotonic saline or choline solution. In 112 mM pure sucrose solution, the wet weight of a single sartorius muscle increased about 30–40% after one hour immersion and then gradually decreased upon further immersion at room temperature (22–23°C). Presumably, the muscle initially gained water and gradually lost it as the intracellular potassium decreased. In spite of the marked change in volume, a large number of muscles were found to maintain resting potentials over 100 mv in sucrose solution up to 16 hours at room temperature. As a routine, a preparation soaked in sucrose solution for a given period, was transferred into Ringer's solution for the purpose of measuring the resting potential. The value of the resting potential dropped in Ringer, but showed no significant difference from the normal value. The resting potential in sucrose sometimes tended to drop when preparations were soaked for more than 10 hours at room temperature. This drop of resting potential seemed to be caused by irreversible deterioration of the muscle membrane; the membrane potential could not be restored even after re-immersion in Ringer's solution for an extended time.

Estimation of potassium content was done immediately after the measurement

TABLE 1

Results obtained from muscles soaked in 112 mM sucrose and Ringer's solutions for 1–3 hours at room temperature

Exp. no.	Soaking sol.	Soaking		Wet weight			Intracellular K-content	Resting potential
		Hours	Temp.	Init.	Final	Rate of change		
				mg	*mg*	%	*mM/ifw*	*mv*
44	Sucrose	1	23	102	138	+35	48.0	93.5±0.8
44	Sucrose	1	23	99	138	+39	47.6	94.9±0.2
44	Sucrose	1	23	92	122	+32.5	53.0	93.0±0.9
46	Sucrose	1	22	89	117	+31.5	71.9	85.6±0.9
46	Sucrose	1	22	93	126	+35	59.4	95.5±0.8
46	Sucrose	1	22	83	107	+29	59.4	94.0±0.8
38	Sucrose	3	23	69	89	+29	66.5	88.5±1.2
38	Sucrose	3	23	67	83	+24	68.1	91.2±1.3
42	Sucrose	3	23	58	74	+27.5	34.4	85.0±1.4
42	Sucrose	3	23	68	78	+15	48.4	93.0±1.0
45	Sucrose	3	23	109	145	+33	50.8	96.5±0.3
45	Sucrose	3	23	105	132	+26	45.8	93.4±0.7
44	Ringer	1	23	69	68	−1.5	105.0	93.6±0.5
46	Ringer	1	22	71	70	−1.4	111.0	98.0±0.3
38	Ringer	3	23	77	75	−2.6	135.0	93.5±0.2
42	Ringer	3	23	72	67	−7	128.0	94.2±0.3
45	Ringer	3	23	75	73	−2.6	135.0	93.5±0.5

TABLE 2

Results obtained from muscles soaked in 112 mM sucrose and Ringer's solutions for 8–10 hours at room temperature

Exp. no.	Soaking sol.	Soaking		Wet weight			Intracellular K-content	Resting potential
		Hours	Temp.	Init.	Final	Rate of change		
				mg	*mg*	%	*mM/ifw*	*mv*
34	Sucrose	8	23	74	82	+11	40.0	97.5±1.3
34	Sucrose	8	23	94	112	+19	43.3	95.6±0.5
34	Sucrose	8	23	92	106	+15	43.2	94.6±1.5
34	Sucrose	8	23	90	109	+21	45.4	99.0±0.8
35	Sucrose	10	22	87	103	+18.5	37.8	84.5±1.2
35	Sucrose	10	22	88	96	+ 9	47.9	101.0±0.9
35	Sucrose	10	22	65	73	+12	42.6	99.5±1.5
35	Sucrose	10	22	66	70	+ 6	45.3	88.5±0.8
34	Ringer	8	23	66	64	− 3	107.0	96.0±0.4
35	Ringer	10	22	83	77	− 7	124.9	93.4±0.5

f resting potential. If muscles were kept
n Ringer's solution for a longer time, the
otassium content apparently increased,
nowing rapid accumulation. The value
f the resting potentials of these muscles,
owever, showed no appreciable change
egardless of the period of immersion in
inger's solution. It was interesting that
ie potassium content of muscles of which
esting potential dropped irreversibly in
ucrose showed no appreciable difference
om those obtained from other muscles.
urthermore, these muscles accumulated
otassium when re-immersed in Ringer's solution although the value of resting potential was not restored.

More than 600 muscles were used throughout the present expermient. It was confirmed that all results could be represented by the values shown in tables 1–3. The intracellular potassium content dropped to less than 20% of the normal value without a marked drop of resting potential after 16–40 hours immersion at different temperatures (table 3).

In some muscles soaked for 16–40 hours, the action potential was recorded after they were transferred into Ringer's

TABLE 3

Results obtained from muscles soaked in 112 mM sucrose and Ringer's solutions for 16–40 hours at 22° and 10°C

Exp. no.	Soaking sol.	Soaking		Wet weight			Intracellular K-content	Resting potential
		Hours	Temp.	Init.	Final	Rate of change		
				mg	*mg*	%	*mM/ifw*	*mv*
36	Sucrose	16	22	96	98	+ 2	11.3	84.1±1.4
36	Sucrose	16	22	79	87	+10	15.9	87.0±1.1
36	Sucrose	16	22	70	77	+10	15.2	87.9±0.8
37	Sucrose	16	22	73	81	+11	27.4	88.6±1.3
37	Sucrose	16	22	93	100	+ 7.5	23.4	84.6±1.5
37	Sucrose	16	22	92	101	+10	17.6	91.0±1.3
51	Sucrose	16	10	218	306	+40	45.7	87.0±1.5
51	Sucrose	16	10	102	152	+49	47.8	85.0±1.9
51	Sucrose	16	10	166	245	+47.5	60.7	87.0±1.6
52	Sucrose	24	10	144	190	+32	23.9	85.6±1.2
52	Sucrose	24	10	113	149	+32	36.2	98.6±1.8
52	Sucrose	24	10	104	120	+15	52.6[1]	95.5±1.0
54	Sucrose	40	10	112	137	+22	21.3	85.0±1.9
54	Sucrose	40	10	83	96	+16	15.5	85.9±0.6
54	Sucrose	40	10	113	141	+25	25.3	88.7±1.6
36	Ringer	16	22	73	73	0	117.0	92.5±0.8
37	Ringer	16	22	78	75	− 4	113.2	96.5±0.5
51	Ringer	16	10	107	110	+ 3	123.3	96.0±0.7
52	Ringer	24	10	155	149	− 4	122.0	95.7±0.6
54	Ringer	40	10	92	86	− 6.5	122.6	92.5±0.7

[1] Muscle from which action potential (fig. 1) was obtained, after soaking in Ringer's solution for 30 minutes.

solution. An example of the action potential is demonstrated in figure 1. In spite of the marked reduction of the intracellular potassium content, these muscles produced practically normal action potentials after 2–3 minutes soaking in Ringer's solution. Typical delayed rectification was observed by applying cathodal square pulses, when the electrotonic potential was recorded in choline Ringer. Thus, neither the falling phase of the action potential in Ringer's solution nor the rectification of the membrane in choline showed a marked alteration in these muscles.

DISCUSSION

Shaw et al. ('56) have pointed out that there is no correlation between the intracellular potassium content and the resting potential of individual muscles in Ringer's solution. Tobias ('50) has obtained comparable results by soaking sartorius muscles in Ringer's solution for periods up to 100 hours at 4–6°C. These experimental evidences, however, may not be sufficient to dismiss the concept of the ionic theory; it may be argued that non-correlation is due to the inaccuracy of the measurement of the potassium content and statistical values of the potential. Results may be more informative in this respect if the intracellular potassium content is altered to a further extent.

Desmedt ('53) soaked muscles in low potassium (0.2 mM) Ringer up to 68 hours at 2–3°C. He found that the intracellular potassium content was reduced to 75% (85–90 mM/ifw) of normal value and the resting potential was 102 mv in 0.2 mM K Ringer. Although he did not mention the potential value observed in Ringer's solution, it is quite likely that he found it to be around 90 mv; a value which does not agree with the prediction of the ionic theory. Adrian ('56) also soaked muscles for 50 hours in low potassium Ringer at 2–3°C and found 44 mM/ifw intracellular potassium content. The potential reversed 25 mv when prepartions were soaked in high potassium solution (75 mM K) and 10 mv in normal sign after 80 minutes corresponding well to the ionic theory. During the course of the present experiment, similar procedures were followed. Four muscles were soaked in sucrose solution for 10 hours (intracellular potassium

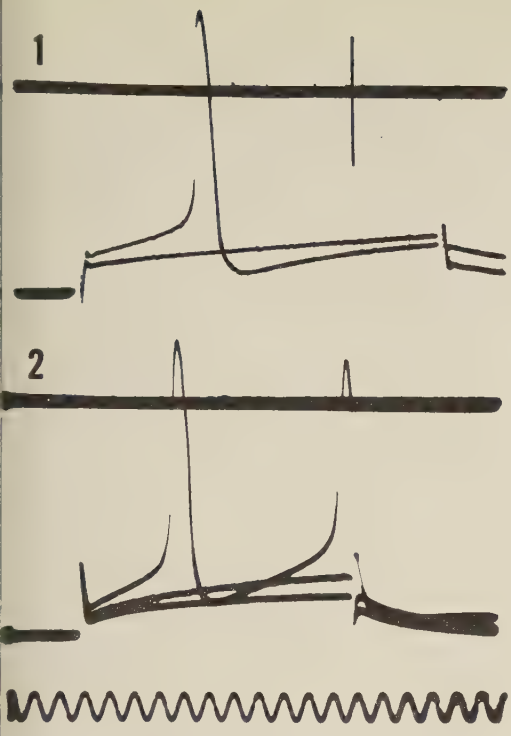

Fig. 1 Record 1 shows action potential obtained in Ringer's solution without any previous soaking procedure. Action potential in record 2 was obtained from muscle which was previously soaked in 112 mM sucrose solution for 24 hours at 10°C and re-immersed in Ringer's solution for about 15 minutes. Internal potassium contents of these muscles are shown in table 3.

content was approximately 40–50 mM/ifw) and then transferred into a solution containing 75 mM potassium. The value of the resting potential was about 16 mv immediately after muscles were transferred into this solution and gradually decreased to about 8 mv after 30–50 minutes. Neither reverse of potential sign nor increase of potential was observed during immersion. This contradiction remains to be solved by further experiments.

The intracellular potassium content could be reduced to a further extent when muscles were soaked long enough in low potassium, potassium-free, or even distilled water (Tobias, '50). When muscles were soaked in various kinds of potassium-free solutions, however, the resting potential dropped gradually, not recovering even after they were re-immersed in normal Ringer's solution. It must be kept in mind that the correlation between the intracellular potassium content and the resting potential should be studied in muscles which membrane has not deteriorated irreversibly. In this respect, hypotonic pure sucrose was the most favorable potassium-free solution. The resting potential of some muscles, however, dropped even in hypotonic pure sucrose and did not recover upon re-immersion in Ringer's solution. These preparations accumulated potassium in Ringer's solution although the resting potential could not be restored, suggesting that there was no relationship between accumulation of potassium and restoration of resting potential; the condition of the membrane seems to be more crucial than potassium accumulation for maintaining resting and action potentials.

The present experimental results do not seem to be favorable for the ionic theory. Further interpretations exceeding the current concept seem to be necessary to explain the establishment of the resting potential.

SUMMARY

1. Frog's sartorius muscles were soaked in 112 mM pure sucrose solution for various periods at different temperatures. Intracellular potassium content was reduced to less than 20% of normal value by this procedure.

2. In spite of the marked loss of intracellular potassium, muscles showed no appreciable reduction of resting potential and produced practically normal action potentials.

3. These results cannot be explained by the current concept based on the ionic theory.

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THE EFFECT OF MULTIVALENT ANIONS ON THE PROTEIN-BOUND AND COMPLEXED CALCIUM IN SERUM[1]

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Electrostatic association between ions ay modify the physicochemical proper-es of solutions containing multivalent ectrolytes. The properties of such solu-ons often exhibit deviations from the ehavior predicted by the Debye-Huckel eory or any of its empirical extensions. n the absence of a more generally applic-ble theory, these deviations are usually xpressed in terms of reversible equilibria etween free ions and ion-pairs (Robinson nd Stokes, '59).

The role of such interactions in biolog-al systems has received scant attention. n other papers in this series it is demon-rated that calcium ions, when electro-atically paired with multivalent anions, re ineffective in sustaining cardiac con-raction in the frog heart (Payne and Valser, '59) and are poorly reabsorbed by he renal tubules (Walser and Browder, 9).

Multivalent ions are present in small mounts in normal plasma; since these ectrostatic complexes are generally eak, the extent of this type of interaction n normal plasma must be slight. In urine, ntracellular fluid, and abnormal plasma, onsiderably larger quantities of multi-alent ions may be present.

In order to estimate the extent of com-lex formation between various ionic spe-ies in these latter fluids, one possible rocedure would be to determine (1) the ree ion concentrations of all cations pres-nt (2) the total concentration of individ-al anions and (3) calculate the amount f each complex from known stability onstants. Binding by undetermined li-ands will then account for the difference etween the total concentration of each cation and the amounts accounted for as free ions and as complexes.

Spectrophotometric methods are now available for measuring free ion concentrations of calcium and magnesium (Walser, '60a). Sodium and potassium (in solutions free of macromolecules) may be assumed to be entirely free, or nearly so. It is therefore possible to test this scheme on protein-free, optically clear, body fluids. This was done by adding multivalent anions to serum, preparing ultrafiltrates, and comparing the observed concentrations of free calcium ions with those predicted from the reported stability constants of the respective ion-pairs. These data are also relevant to the roles of ionic strength and of ion-pair formation in modifying the protein-binding of calcium in serum.

METHODS

Venous blood was obtained from normal human subjects or dogs, and allowed to clot. Serum was separated without exposure to air and stored at −10°C until use. In each experiment, several mixtures were prepared consisting of a constant volume of serum diluted to a constant extent by two salt solutions added in varying proportions (see table 1). One salt solution generally consisted of sodium chloride and the other of the sodium salt of the anion under study. These mixtures were bubbled with 3% carbon dioxide for 15 minutes (at room temperature) and placed in sacs of Visking tubing. Each sac was placed in an ultrafiltration appa-

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ratus as described by Toribara, Terepka, and Dewey ('57), which was then filled with 5% carbon dioxide. After 5 hours centrifugation at 36–38°C the clear protein-free ultrafiltrates were removed. pH was promptly determined at room temperature using a Beckman pH meter. Calcium was determined in one control serum sample and in each ultrafiltrate. Free calcium ion concentration and the concentration of the anion under study were determined in each ultrafiltrate.

Analytical methods were as follows: calcium, oxalate precipitation and permanganate titration (Clark and Collip, '25); free calcium ions, murexide (Walser, '60a); phosphate, Fiske and Subbarow ('25); citrate, preliminary acid digestion and color development according to Natelson, Pincus, and Lugovoy ('48) conversion to pentabromacetone according to Taylor ('53); ferrocyanide was determined by treating trichloroacetic acid filtrates with hydrogen peroxide to form ferricyanide, and measuring optical density at 415 mu. Sulfate was not measured; it was assumed that normal dog plasma contains 10^{-3} M sulfate and that added sulfate distributed between ultrafiltrate and plasma water in a ratio of 0.95 (Swan, Feinstein, and Madisso, '56).

Ionic strength of the salt solutions was calculated assuming complete dissociation of all the salts studied, with the exception of sodium ferrocyanide solution, 0.06 M; the ionic strength of this solution was taken as 0.5 M rather than 0.6 M to allow for incomplete dissociation; no value for this dissociation constant could be found. Ionic strength of normal serum and serum ultrafiltrate was taken as 0.16 M.

In two experiments the solutions added in varying proportions consisted of water and sodium chloride solutions, in order to determine the effect of ionic strength upon protein-binding of calcium.

In additional experiments, salt solutions were added directly to serum ultrafiltrate; in these experiments, calcium was determined on an aliquot of ultrafiltrate and free calcium ion concentration on each mixture; anion concentration was obtained by calculation.

RESULTS

Thiocyanate, perchlorate, and nitrate when substituted for chloride added directly to ultrafiltrate were without effect on calcium ion concentration. This is to be expected, since these univalent anions do not complex calcium appreciably (Bjerrum, Schwarzenbach, and Sillen, '58).

Sodium ferrocyanide, 0.016 M, substituted for sodium chloride, 0.16 M, added to serum led to a modest decline in calcium ion concentration (table 1). The ultrafiltrable calcium remained nearly constant. This increment in complexed calcium presumably represents the calcium ferrocyanide ion-pair, which has a net negative charge of two. In order to achieve higher concentrations of ferrocyanide, without further dilution of the serum, a more concentrated saline solution (0.5 M) was added to the control sample serum, and a more concentrated ferrocyanide solution (0.06 M) was substituted. The resulting ionic strength of the serum ultrafiltrate (assuming 0.16 to be the normal value) is calculated to be 0.22. As shown, a more pronounced change in complexed calcium is observed.

Ultrafiltrable calcium showed some decrease in this experiment, suggesting that the calcium ferrocyanide ion-pair may be more firmly found to plasma protein than is free calcium. This possibility is borne out by the fact that ferrocyanide failed to distribute itself across the membrane in accordance with theoretical expectations implying some protein-binding. Similar conclusions were reached by Kleeman and Epstein ('56) working with isotopic ferrocyanide. The proximity of binding sites for calcium and ferrocyanide on the protein molecule may permit the ion-pairs themselves to become bound, or, stated differently, the calcium and ferrocyanide ions associated electrostatically with protein may also become associated with one another.

Two experiments with sulfate are shown. Ultrafiltrable calcium was unaffected but complexed calcium increased. Again, the failure of protein-bound calcium to fall *pari passu* with ionic calcium suggests some protein-binding of the calcium sulfate ion-pairs.

In the case of phosphate, the concentration of the complexing anion HPO_4^{2-} was calculated from total inorganic phosphate (shown in the table) and pH, using pK = 6.8. Complexed calcium again in

TABLE 1

Analysis of ultrafiltrates of serum diluted with various salt solutions

Mixture analyzed					Mixture		Ultrafiltrate		
Serum	Sol'n I	Sol'n II	μ	pH of UF	[Ca]	[L]	[Ca]	[L]	$[Ca^{2+}]$
ml	*ml*	*ml*	*M*		*mM*	*mM*	*mM*	*mM*	*mM*
I. Sol'n I: NaCl, 0.016 M; Sol'n II: $Na_4Fe(CN)_6$, 0.006 M.									
10	2.0	0.0	*0.16*	7.41	2.19	*0.00*	1.55	0.00	1.43
10	1.5	0.5	*0.16*		2.19	*0.67*	1.54	0.64	1.40
10	1.0	1.0	*0.16*		2.19	*1.34*	1.55	1.36	1.31
10	0.5	1.5	*0.16*		2.19	*2.00*	1.56	2.08	1.26
10	0.0	2.0	*0.16*	7.41	2.19	2.67	1.51	2.53	1.22
II. Sol'n I: NaCl, 0.5 M; Sol'n II: $Na_4Fe(CN)_6$, 0.06 M.									
10	2.0	0.0	*0.22*	7.40	2.05	*0.00*	1.68	0.00	1.41
10	1.5	0.5	*0.22*		2.05	*2.50*	1.63	2.47	1.34
10	1.0	1.0	*0.22*		2.05	*5.00*	1.63	4.85	1.16
10	0.5	1.5	*0.22*		2.05	*7.75*	1.56	7.83	1.01
10	0.0	2.0	*0.22*	7.38	2.05	*10.00*	1.56	10.05	0.92
III. Sol'n I: NaCl, 0.16 M; Sol'n II: Na_2SO_4, 0.053 M, added directly to ultrafiltrate									
3[1]	1.0	0.0	*0.16*	7.38			1.27	*1.00*	1.05
3[1]	0.5	0.5	*0.16*				1.27	*7.63*	0.91
3[1]	0.0	1.0	*0.16*	7.38			1.27	*14.25*	0.81
IV. Sol'n I: NaCl, 0.5 M; Sol'n II: Na_2SO_4, 0.17 M									
6	2	0	*0.22*	7.32	1.90	*1.0*	1.36	*1.1*	1.16
6	1	1	*0.22*	7.40	1.90	*22.2*	1.42	*24.8*	0.86
6	0	2	*0.22*	7.38	1.90	*43.5*	1.43	*48.6*	0.76
V. Sol'n I: NaCl, 0.16 M; Sol'n II: Na_2HPO_4, 0.05 M, NaH_2PO_4, 0.012 M									
8	2.0	0.0	*0.16*	7.42	1.94	0.97	1.24	0.88	1.23
8	1.5	0.5	*0.16*	7.43	1.94	4.05	1.25	4.07	1.12
8	1.0	1.0	*0.16*	7.35	1.94	7.13	1.24	7.36	1.02
8	0.5	1.5	*0.16*	7.37	1.94	10.21	1.11	10.45	0.88
8	0.0	2.0	*0.16*	7.37	1.94	13.29	1.00	13.30	0.65
VI. Sol'n I: NaCl, 0.16 M; Sol'n II: Na_3Citrate, 0.0125 M, NaCl, 0.08 M									
10	2.0	0.0	*0.16*	7.33	2.05	0.14	1.57	0.14	1.48
10	1.5	0.5	*0.16*		2.05	*0.66*	1.60	0.63	1.24
10	1.0	1.0	*0.16*		2.05	*1.18*	1.71	1.22	1.13
10	0.5	1.5	*0.16*		2.05	*1.70*	1.78	1.56	0.95
10	0.0	2.0	*0.16*	7.32	2.05	2.22	1.77	2.10	0.84
VII. Sol'n I: H_2O; Sol'n II: NaCl, 1.6 M									
3	1.00	0.00	*0.12*		2.19		1.21		
3	0.90	0.10	*0.16*		2.19		1.47		
3	0.84	0.16	*0.18*		2.19		1.48		
3	0.69	0.31	*0.24*		2.19		1.70		
3	0.00	1.00	*0.52*		2.19		1.83		
VIII. Sol'n I: H_2O; Sol'n II: NaCl, 0.4 M									
3	1.00	0.00	*0.12*		1.91		1.22		
3	0.60	0.40	*0.16*		1.91		1.38		
3	0.25	0.75	*0.195*		1.91		1.48		
3	0.00	1.00	*0.22*		1.91		1.47		

μ = Ionic strength.
[Ca] = Measured calcium concentration.
[L] = Concentration of anion under study.
$[Ca^{2+}]$ = Measured free calcium ion concentration.
[1] Volume of ultrafiltrate used.
Calculated (or assumed) values are italicized.

creased. In the last two samples, ultrafiltrable calcium fell sharply. This may reflect the formation of microcrystals of $CaHPO_4$ which fail to pass through the cellophane membrane. The ratio of ultrafiltrate phosphate to whole serum phosphate approximated unity in all except the first sample, diluted only with saline, in which it was 0.91. It is not certain whether the reduction of ultrafiltrable calcium in this experiment reflects microcrystal formation or increased protein-binding of the calcium phosphate ion-pair. However, the increase in *complexed* calcium is almost certainly due to complex formation, rather than to the formation of microcrystals in the ultrafiltrate.

For comparison with these effects of ion-pair formation, an experiment (VI) was conducted employing citrate, which forms a chelate complex with calcium. With increasing citrate concentration, calcium ion concentration and protein-bound calcium both decline, as anticipated from earlier observations (Shelling and Maslow, '28; Hastings et al., '34). If protein-bound calcium is calculated as total calcium in the mixture minus ultrafiltrate calcium (ignoring corrections for water content and Donnan effect, which are similar in magnitude and opposite in direction for bivalent cations), the ratios of protein-bound to free calcium ion concentration in the 5 samples are 0.32, 0.36, 0.30, 0.28, and 0.33, respectively. Thus protein - bound calcium falls approximately in proportion to free calcium ions upon the addition of citrate.

We have reported (Walser and Browder, '59) a decrease in protein-binding of calcium in dogs given large amounts of sodium sulfate intravenously and have also made similar (unpublished) observations on calcium, magnesium, and radiostrontium binding during infusion of sulfate or ferrocyanide. In the present experiments, addition of these salts at constant ionic strength failed to reduce protein-binding of calcium. Therefore the possibility that increased ionic strength itself was responsible for the changes reported was investigated. Varying concentrations of sodium chloride were added to serum and ultrafiltrable calcium was determined. As indicated in the table, protein-bound calcium is quite sensitive to increases in ionic strength. These results provide an explanation for the change in protein-binding of alkaline earth cations during infusion of sodium salts of multivalent anions.

The observed changes in calcium ion concentration were compared with those predicted from physicochemical constants as follows. Dissociation constants, K, of 0.00017 M, 0.006 M, and 0.003 M for $CaFe(CN)_6^{2-}$, $CaSO_4$, and $CaHPO_4$ respectively (at zero ionic strength and 25°C) were selected from those given in the literature (Bjerrum et al., '58) as being the most reliable. These were corrected to the ionic strength employed in each experiment by the use of a general activity coefficient equation (Davies, '38). Free calcium ions, $[Ca^{2+}]$ were calculated from the equation (Hastings et al., '34)

$$[Ca^{2+}] = \frac{[Ca] - [Ca][L]K - \sqrt{([Ca][L]K)^2 - 4[Ca][L]}}{2}$$

where [Ca] represents the ultrafiltrable calcium minus the normal amount of complexed calcium (as determined from the control sample diluted only with saline) and [L] represents the measured concentration of the anion added ($Fe(CN)_6^{4-}$, SO_4^{2-} or HPO_4^{2-}). Competition between calcium and other cations for these ligands was ignored.

The correspondence between observed $[Ca^{2+}]$ and that calculated from this equation is shown in figure 1. Although the use

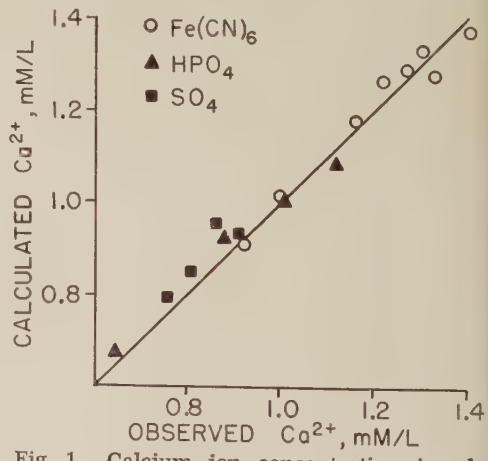

Fig. 1. Calcium ion concentration in ultrafiltrates of serum diluted with sodium sulfate, phosphate, or ferrocyanide as compared with values predicted from reported dissociation constants.

f alternative activity coefficient equations or the selection of different constants in ne equation employed) would lead to dif-erent values, the equation used herein is ne same as that on which most of the hysicochemical data cited are based.

DISCUSSION

Several inferences may be drawn from nese observations. First, it is apparent nat the murexide method does not meas-re calcium ions which are electrostati-ally paired with multivalent anions. The resence of the associated anions evi-ently prevents calcium from combining ith murexide. Presumably calcium in nese ion-pairs is still ionized. It follows nat the murexide method (in common ith the frog heart method (Payne and Valser, '59) determines only free calcium ons, rather than all of the ionized calcium.

Secondly, these observations show that levation of the plasma concentration of ultivalent anions may lead to a signifi-ant reduction in free calcium ion concen-ation. Similar plasma concentrations an be produced in intact animals, and, in ne case of sulfate and phosphate, may ccur normally in the urine.

The major inference to be drawn relates the analysis of complex electrolyte mix-ures such as urine. The data presented ffer partial support for the concept that ndividual complexes can be quantified by ne method outlined; namely, determina-on of free calcium ions, total anion con-entrations, and application of known dis-ociation constants. Further studies of ixtures of several anions, together with nalysis of magnesium ion concentrations, re also indicated.

Finally it should be noted that ion-pair ormation does not result in reduced pro-ein-binding of calcium, as might be ex-ected. These electrostatic complexes of alcium appear to be bound to plasma pro-ein at least as strongly as are free calcium ons. This phenomenon represents a dis-nct difference between these electrostatic omplexes and chelate compounds such s calcium citrate.

Protein-binding of phosphate in normal lasma is not dependent upon the presence f calcium or magnesium (Walser, '60b). et elevation of plasma calcium may lead an increase in nondiffusible phosphate (Grollman, '27; Hopkins, Howard, and Eisenberg, '52). This effect may be analogous to the increase in nondiffusible calcium observed herein when phosphate was elevated. Electrostatic interaction between small oppositely charged ions may thus augment their binding to protein, but is certainly not essential to it.

SUMMARY

Sodium ferrocyanide, sulfate, or phosphate (pH 7.4) were substituted in varying amounts for sodium chloride in diluted serum.

At constant ionic strength, all three salts led to either an increase or no change in protein-bound calcium and reductions in free calcium ion concentration corresponding closely to values predicted from reported dissociation constants of the electrostatic complexes $CaFe(CN)_6^{2-}$, $CaSO_4$, and $CaHPO_4$.

The increase in protein-binding of calcium produced by these anions contrasted with citrate, which led to a decrease.

ACKNOWLEDGMENTS

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revention of the Golgi Response of Intestinal pithelial Cells by Monoiodoacetic Acid in ats Fed Fat or Injected with Chyme from at-fed Donor Animals[1]

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In view of the striking response of the olgi apparatus of intestinal epithelial lls of the rat to the absorption of fat Adamstone, '59; Adamstone and Taylor, 9), it appeared highly desirable to de- rmine whether the injection of monoiodo- cetic acid (MIA) could block this reac- on, particularly in view of its wide use an inhibitor of fat absorption and en- me action (Verzar and Laszt, '34).

Experiments of this sort which were rried out by Verzar and co-workers and which fat was fed *per os* were regarded them (see Verzar, '36) as furnishing ear evidence that fat absorption does not cur following administration of MIA, al- ough their data showed that much of the t was still present in the stomach after hours. In other experiments fat was in- cted directly into a loop of the intestine both normal and MIA-injected rats. In th cases there was very little fat absorp- n and it was concluded that the failure fat absorption was not due to delayed nptying of the stomach but to the inhibi- ry effect of MIA. Frazer ('48) repeated ese experiments but was unable to con- m Verzar's conclusions and also empha- zed the toxic action of the drug and the olonged retention of fat in the stomach.

In the present tests (experiment I, be- w) we have repeated the procedures of erzar and Frazer. In addition, we have tempted (in experiment II) to circum- nt the retention of fat in the stomach, d the consequent failure of fat absorp- n in epithelial cells of the intestine, by e direct transfer of digesting fat from e gut of a normal animal to the gut of an jected one. Thus it has been possible to termine whether a Golgi response is ob- tained in intestinal epithelial cells of the experimental recipient animal which has been injected with MIA and in which fat was available for absorption.

Experiment I

MATERIALS AND METHODS

In this set of experiments (table 1) 12 rats, starved for 24 hours were used and monoiodoacetic acid (MIA) at levels ranging from 0.02 to 0.08 mg/gm body weight was injected at varying times prior to feeding. Each rat was fed 0.1 or 0.25 cm^3 olive oil colored with Sudan IV, 25 minutes before sacrifice (45 minutes in one rat). In the first three animals the MIA was injected intramuscularly into the right leg and in the remaining 9 animals the injections were given intraperitoneally.

At autopsy, tissue samples were secured from the duodenum, upper and lower jejunum and ileum. A small piece of each sample was kept in Ringer's solution for examination in the fresh condition and the remainder was fixed in Lavdowsky's F.A.A. for later preparation of hematoxylin stained paraffin sections. A small piece of the fresh material was teased out in a drop of Ringer's solution on a microscope slide and the cells examined as a fresh mount to determine whether a prominent Golgi apparatus could or could not be seen. The cells were designated as positive if a Golgi apparatus could be seen and negative if it was not apparent. A percentage count was made of positive and negative cells at each level of the gut.

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[2] With technical assistance of Ralph A. Jersild.

TABLE 1

Response of Golgi apparatus of intestinal epithelial cells of rats following the injection of monoiodoacetic acid and the feeding of fat

Animal no.	Monoiodoacetic acid		Fat		Cell count							
	Amount	Time prior to feeding fat	Olive oil	Time prior to sacrifice	Duodenum		Upper jejunum		Lower jejunum		Ileum	
					−	+	−	+	−	+	−	+
	mg/gm	*min.*	*cm³*	*min.*								
1[1]	0.08	60	0.10	25	19	81	62	38	88	12	95	5
2[1]	0.08	120	0.10	25	95	5	91	9	90	10	98	2
3[1]	0.08	120	0.25	25	38	62	27	73	70	30	94	6
4	0.08	60	0.10	25	95	5	99	1	91	9	90	10
5	0.08	120	0.25	25	96	4	95	5	92	8	97	3
6	0.08	60	0.10	45	99	1	91	9	98	2	95	5
7	0.08	60	0.25	25	94	6	95	5	97	3	91	9
8	0.08	60	0.25	25	99	1	91	9	98	2	95	5
9	0.02	60	0.25	25	47	53	68	32	96	4	96	4
10	0.04	60	0.25	25	95	5	98	2	95	5	99	1
11	0.06	10	0.10	25	97	3	98	2	95	5	100	0
12	0.06	10	0.10	25	97	3	96	4	94	6	94	6

[1] Intramuscular, all others intraperitoneal.

OBSERVATIONS

The injection of large doses of MIA was followed quickly by rather violent reactions including imbalance, prostration and labored breathing. In a few cases death occurred. At autopsy, marked hemostasis and vasodilation were evident throughout the entire length of the gut (figs. 10, 11) and both stomach and intestine were full of a watery fluid. In the three animals which had been injected intramuscularly, the site of injection was found to be highly edematous. Furthermore, in these animals it was observed that a small amount of fat had passed into the intestine, whereas in the other nine it had been entirely retained in the stomach. In earlier experiments with normal animals (Adamstone, '59), it was found that ingested oil had passed from the stomach into the intestine 15 minutes after feeding.

Condition of the Golgi apparatus and cell counts in fresh cells. Examination of the fresh cells showed that in two of the three animals injected intramuscularly, the Golgi apparatus could be seen in a considerable number of cells from both duodenum and upper jejunum. As usual, it appeared as a transverse dark band in the cell above the nucleus and was usually associated with vacuoles of absorbed fat. In all the remaining animals which had received an intraperitoneal injection (except one), the Golgi apparatus was inconspicuous or entirely lacking at all levels of the gut (fig. 1). Animal 9 (table 1), which had received the smallest dose of MIA, was an exception readily explainable on the basis of the small amount of MIA received. Data relating to these studies and the percentage counts of positive and negative cells are given in table 1.

Cytological observations on fixed stained sections. As a result of former studies on fat-fed animals, the study of hematoxylin stained sections was centered largely on the presence or absence of the negative image of the Golgi apparatus. The histological picture presented by the sectioned material in relation to this organelle parallels quite closely the findings in the study of fresh material. Thus, in sections of material from the duodenum and jejunum of animals 1 and 3 there was a strong negative image of the Golgi apparatus in many cells and it was also visible in the material from the upper jejunum of animal 9. In animals 1, 2 and 3, the negative image of the Golgi apparatus was also quite prominent in the lower jejunum and ileum where there was considerable debris. In all other material examined the structure was generally lacking (fig. 2) except for its occasional occurence in small restricted areas.

SUMMARY

The almost complete retention of the ingested fat in the stomach of the animals used in these experiments and its consequent failure to reach the intestinal epith

l cells, where absorption might occur, ould appear to throw considerable doubt this type of experiment as a method of termining the effect of MIA on fat abrption or on the response of the Golgi paratus. Moreover, the edematous conion of the injection site noted in animals 2 and 3 could well limit the access of IA to the circulatory system, thus result- g in its apparent ineffectiveness in block- g fat absorption in these animals. In ani- al number nine the inffectiveness of the IA was probably due to the small dosage sed.

Experiment II

MATERIALS AND METHODS

In the second series of tests (table 2) it as decided to adopt the technique of yme transfer (Adamstone and Taylor, 9), whereby chyme from a fat-fed animal, which a positive Golgi response had al- ady occurred, was transferred to the gut a recipient animal injected with MIA. ese earlier experimental results obtained ith normal animals will thus serve as a ntrol for the present experimental group. e starved donor animals were fed 0.5 cm^3 olive oil colored with Sudan IV and minutes later when sacrificed the yme was removed from the gut for trans- r. The recipients were injected intra- itoneally with doses of MIA ranging om 0.02 to 0.07 mg/gm body weight, 45 inutes before chyme transfer. The arved recipient was anaesthetized with mbutal and 0.1 cm^3 chyme injected into ligated segment of the jejunum where it as allowed to remain 25 minutes.

At autopsy the same general responses MIA were observed in these animals as the preceding group, viz., hemostasis d vasodilation in the walls of the gut d in the mesenteries and the accumula- n of watery fluid in both stomach and testines. At this time tissue samples were moved and placed in Ringer's solution r study in the fresh condition, and in A.A. for fixation and later preparation of matoxylin-stained sections. Tissues were moved from experimental areas of the t of both donor and recipient, and con- l material was secured from the recipi- t both before chyme transfer and at the mination of the experiment. The con- l material taken before chyme transfer was removed as a small bleb of tissue from the actual site of injection so that a comparison could be made of conditions in the gut in the actual experimental area.

OBSERVATIONS

Condition of the Golgi apparatus and cell counts in fresh cells. Study of fresh epithelial cells teased out from donor tissues in Ringer's solution, showed, in every case, a striking dark zone above the nucleus which was resolved into the typical Golgi canals on examination with the oil immersion objective of the microscope, (figs. 3, 4). Cell counts showed a great preponderance of positive cells in all animals (table 2).

In the recipient animals by contrast, no Golgi apparatus was visible in most cells. Negative cells predominated in both samples of control material and in all experimental material from injected areas (figs. 6, 8; table 2) except in the two receiving less than 0.06 mg/gm. In these there were some positive cells.

Cytological observations on fixed stained material. In sections of the various tissue samples stained with Heidenhain hematoxylin the donor material (fig. 5) presented a strong negative image of the Golgi apparatus paralleling the predominantly positive picture seen in the fresh material (table 2). In sections of material from recipient animals (fig. 9), however, a negative image was generally lacking except in specimens 1 and 2 in which there was considerable variation in the cells at different levels of individual villi. In control material (fig. 7), the negative image was almost entirely lacking. Thus, conditions in the stained material from both donor and recipient again paralleled those in fresh cells.

SUMMARY

In the second group of experiments there can be no question that material which could normally elicit a positive Golgi response was present in contact with absorptive cells of the intestinal epithelium of the recipient animals. The cell counts, however, demonstrate that when MIA was administered in sufficiently strong dosage there was very little fat absorption and practically no response of the Golgi apparatus.

TABLE 2

Response of Golgi apparatus of intestinal epithelial cells of rats to chyme from fat-fed donor after intraperitoneal injection of monoiodoacetic acid

Animal	Donor				Recipient									
					Monoiodoacetic acid		Chyme		Cell count					
	Fat fed	Time in gut	Cell count		Amount	Time before chyme trans.	Amt.	Time in gut	Control before		Experiment		Control after	
			−	+					−	+	−	+	−	+
	cm³	*min.*			*mg/gm*	*min.*	*cm³*	*min.*						
1	0.5	45	3	97	0.02	45	0.1	30	96	4	95 87 56	5 13 44	98	2
2	0.5	45	3	97	0.04	45	0.1	30	97	3	65 73	35 27	100	0
3	0.5	45	2	98	0.06	45	0.15	30	94	6	89 96	11 4	97	3
4	0.5	45	1	99	0.06	45	0.1	30	97	3	96 99	4 1	100	0
5	0.5	45	2	98	0.06	45	0.1	30	94	6	98 100	2 0	99	1
6	0.5	45	0	100	0.06	45	0.1	30	95	5	95 92	5 8	97	3
7	0.5	45	5	95	0.06	45	0.1	30	99	1	99 95	1 5	97	3
8	0.5	30	3	97	0.06	15	0.1	30	96	4	95 96	5 4	98	2
9	0.5	45	1	99	0.07	45	0.1	30	97	3	97 98	3 2	92	8
10	0.5	45	9	91	0.07	15	0.1	20	97	3	83 89	17 11	99	1

DISCUSSION

From the findings recorded above it is early evident that the injection of MIA rior to feeding fat, as was done in experi- ent I, cannot be regarded as giving un- quivocal evidence concerning the relation- ip between the absorption of fat and the ccurrence of the prominent Golgi re- ponse which was encountered in earlier udies on fat absorption in the rat. It ould appear, therefore, that the pro- nged retention of fat in the stomach and e severe toxic effects of the drug, cause n almost complete lack of absorbable fat the intestine as emphasized by Frazer. his amply explains the lack of fat ab- rption as well as the failure of the Golgi sponse which we have described.

In the second set of experiments de- ribed herein, the harmful effects of the rug were still encountered. However, ilure of the ingested fat to pass into the t was no longer a factor either in fat sorption or in the Golgi response since yme capable of producing both effects as injected directly into the gut of the perimental animal. Even under these rcumstances there has been little or no idence of fat absorption and the Golgi sponse, which occurred without failure earlier experiments involving chyme ansfer to normal untreated rats (Adam- one and Taylor, '59), was not encoun- red in any animal in which an adequate se of MIA was administered.

From the results of these experiments is not yet clear whether MIA has (1) a irect effect on the Golgi apparatus, as for xample, in inhibiting a possible chemical ctivity related to fat absorption, or (2) hether the Golgi response fails to occur merely because fat has not been absorbed. This second effect might be caused by some effect on the plasma membrane or by interference with some other functional cellular activity (exclusive of the Golgi apparatus) associated with fat absorption.

CONCLUSIONS

The use of monoiodoacetic acid to prevent the response of the Golgi apparatus following the ingestion of fat has been tested by feeding olive oil to animals injected with MIA and by the transfer of fat-induced chyme from an untreated animal to the gut of an animal injected with MIA. The results of these experiments indicate that a Golgi response fails to appear in the fat-fed animals but the test is not conclusive because fat fails to leave the stomach. However, following transfer of fat-induced chyme to a recipient animal injected with monoiodoacetic acid, the Golgi response again fails to appear. The reaction, therefore, may be a direct effect on the Golgi apparatus or it may be that the Golgi response fails to occur because fat absorption is affected in some unknown manner.

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PLATE 1

EXPLANATION OF FIGURES

1, 2 Intestinal epithelial cells from duodenum of fat-fed rat injected with MIA. The Golgi apparatus cannot be seen in fresh cells (fig. 1) and the negative image is lacking in hematoxylin-stained sections (fig. 2). × 950.

3 Fresh cells from jejunum of donor rat fed 0.5 cm^3 olive oil 45 minutes before sacrifice. The Golgi apparatus is prominent in these cells as a dark band but is resolved into vesicles in cell at right. × 950.

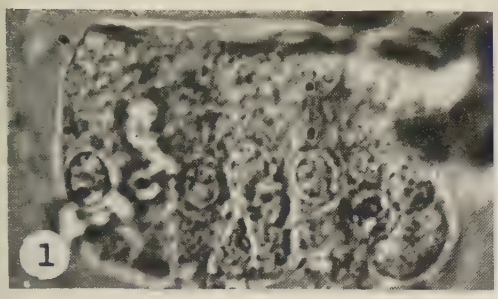
4, 5 Cells from intestine of donor fed as above. In fresh cells (fig. 4) the Golgi apparatus forms a band of vesicles above the nuclei of the cells, and in hematoxylin-stained sections (fig. 5) it forms a prominent negative image. × 950.

6, 7 Cells of fresh (fig. 6) and fixed stained material (fig. 7) from bleb of control tissue of recipient removed at time of chyme transfer show no indication of the Golgi apparatus or the negative image. × 950.

8, 9 Intestinal epithelial cells from injected segment of recipient rat 30 minutes after chyme transfer. Fresh cells (fig. 8) do not show the Golgi apparatus and fixed stained sections lack a negative image (fig. 9). × 950.

10 Loop of intestine of MIA-injected rat showing pronounced vasodilation in mesentery and wall of intestine as well as marked muscular constrictions.

11 Fixed stained section of villus from intestine of MIA-injected rat showing great vasodilation.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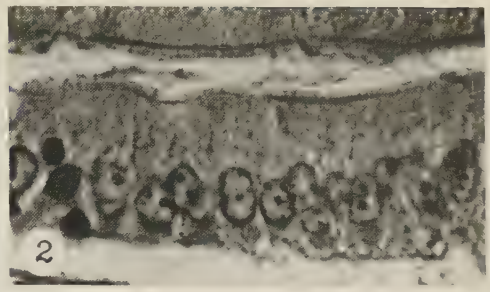
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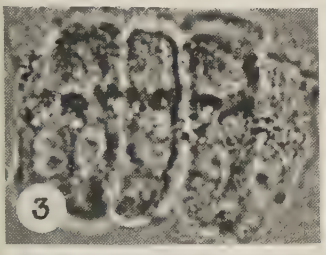
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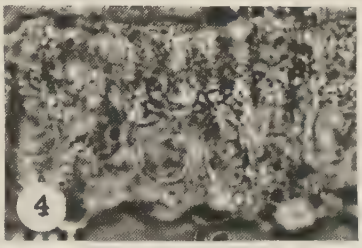
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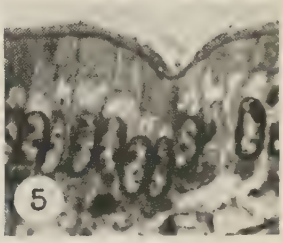
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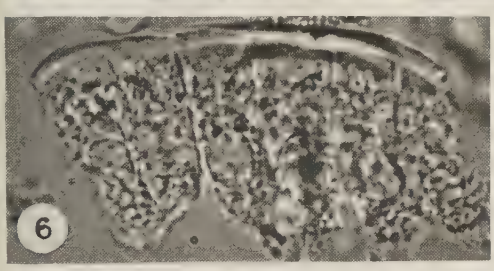
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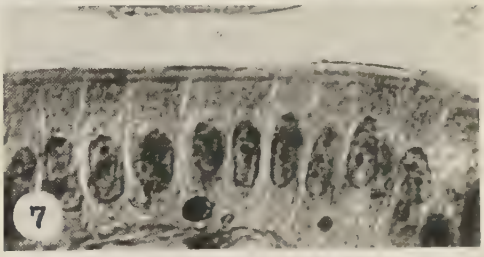
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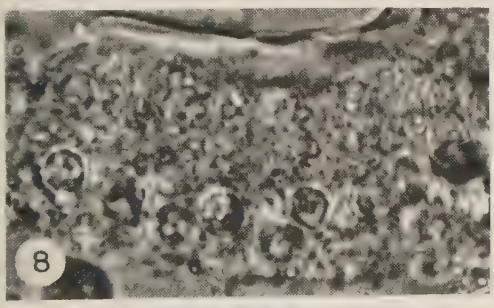
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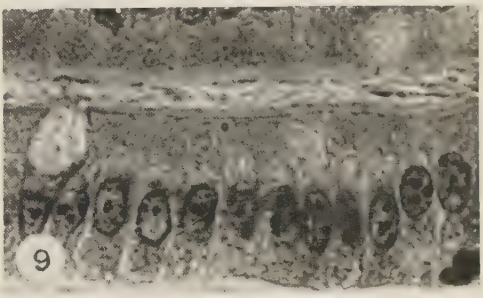
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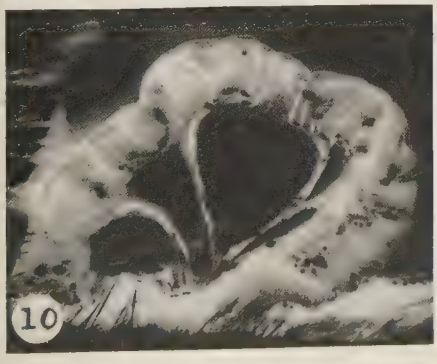
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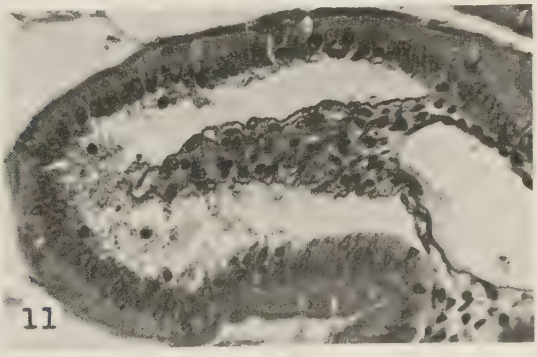
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The Locus of the Electromotive Force in Frog Skin[1]

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The isolated skin of the frog has become a classical subject for studies of active ion transport and the related bioelectric potential. Most recent discussions of this system have assumed that the transport process, and hence the electromotive force, is localized in the region of the *stratum germinativum* of the epidermis. This locus was suggested on logical grounds by Ussing ('48), and the studies of Ottosen et al. ('53) and Engbaek and Hoshiko ('57) appeared to confirm his view. Evidence, largely from this laboratory (Fleming, '56, '57) showed that removal of the *tela subcutanea*, a thin cellular layer separating the *stratum compactum* of the dermis (corium) from the subcutaneous lymph spaces (Gaupp, 1899), abolishes most of the skin potential and the active transport, and increases the permeability of the skin to ions and to non-electrolytes. Crisera ('56), using a dye technique for marking the position of an electrode in the skin, found evidence of a potential drop across the *tela subcutanea* amounting to as much as 50% of the skin potential in a small number of cases. Koblick ('57, '58) found that most of the cholinesterase of the skin is localized in the *tela;* cholinesterase has been implicated in the transport process (Kirschner, '53), and Koblick ('59) has developed a theory of active transport in which cholinesterase plays a significant role. All of the above observations suggest that the *tela subcutanea* may have some important part in both active transport and skin potential, and have prompted us to reexamine with care the nature of the potential field in the skin.

MATERIALS AND METHODS

The observations were made on belly skin of the frog *Rana pipiens* obtained from commercial suppliers in Minnesota or Wisconsin, and kept in running tap water aquaria until use. Frogs were killed by decapitation, and skinned. The cleaned belly skin was placed in our standard saline solution, 50 mM NaCl, 5 mM KCl, for a period of equilibration, and then mounted in one of two ways. In some experiments, the skin was gently stretched over a perforated polyethylene disk, and clamped in place with a close-fitting plexiglass ring which was part of a chamber separating two bodies of aerated saline. In other experiments, the skin was similarly stretched over an ordinary rubber stopper with a single center hole, and smaller perforations distributed evenly over the surface. A cylindrical upper chamber was fitted tightly over the skin, and the surface of the skin next to the rubber stopper was irrigated from below through the central hole with aerated saline. The latter system proved most satisfactory in avoiding polarization effects and the irregularities in skin potential caused by the mechanical action of air bubbles striking the skin surface.

Potential measurements were made with an oscilloscope (Tektronix type 512) or a recording potentiometer (Leeds and Northrop Speedomax). An indifferent Ag-AgCl electrode in glass with a relatively large tip diameter, and filled with 3 M KCl saturated with AgCl, was placed in the lower chamber of the plexiglass apparatus, or in contact with the irrigating fluid of the rubber stopper apparatus. A microelectrode of the same composition, with a tip diameter of about 10 μ and a resistance of three to 20 megohms, was mounted on a micrometer screw arranged so that the direction of electrode travel was approximately normal to the skin surface, and so that the tip of the electrode was directly above one of the perforations in the sup-

[1] This work was supported by a grant B-123 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, U. S. Public Health Service.

porting disk or stopper. The potential was led off from the microelectrode through a cathode follower to the recording instrument.

For measurements, the electrode was moved by hand to a position close to the skin surface. To avoid the irregularities introduced by manual operation of the electrode movement, the electrode was driven through the skin mechanically. For oscilloscope measurements, the driving de vice was that shown in figure 1. This consisted of a pulley connected to the micrometer screw and operated by a falling weight. The weight was floated in a tube filled with mercury. The tube was provided with a drain closed by a stopcock and a pinch clamp. The mercury outflow and hence the rate of movement of the electrode, could be adjusted to any de

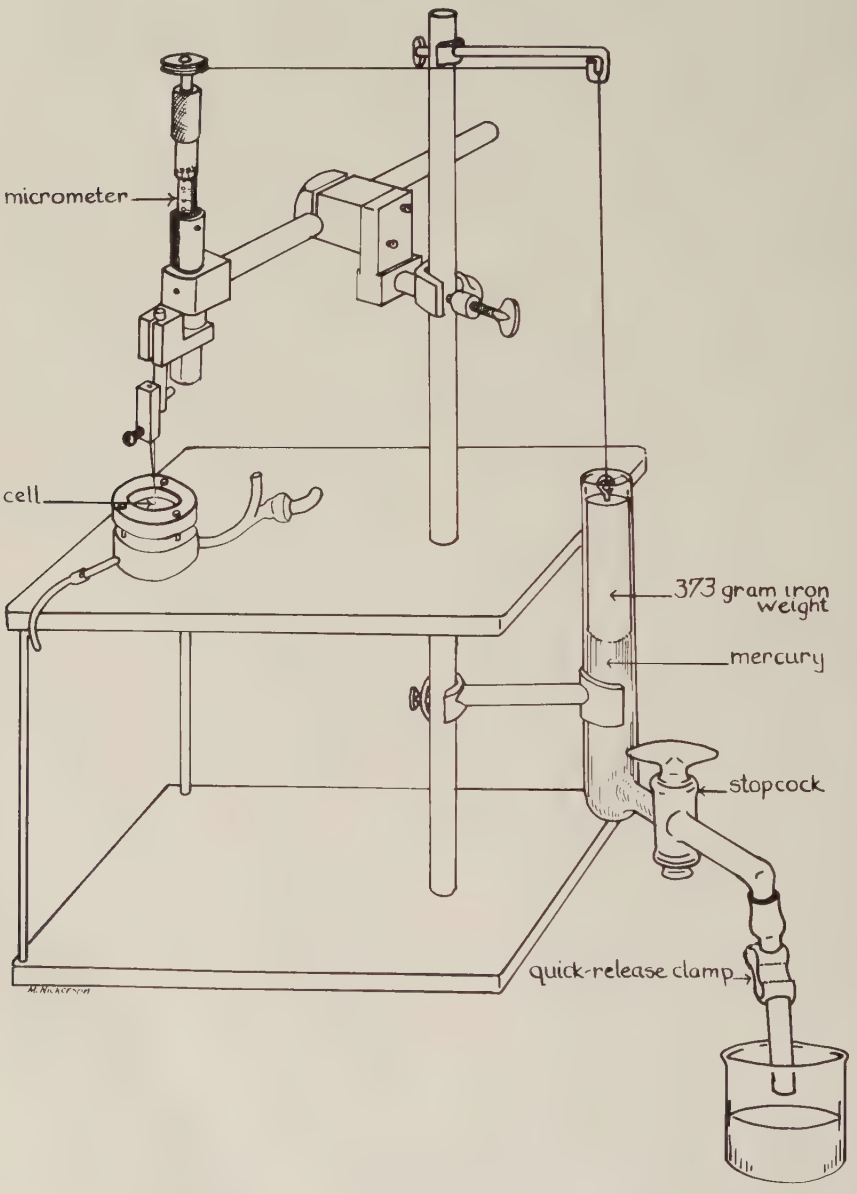

Fig. 1 Apparatus used for mechanical propulsion of an electrode through frog skin.

sired rate by adjustments of the stopcock, and could be stopped and started by closing or opening the pinch clamp. For measurements with the recording potentiometer, the micrometer was driven by a cam attached to a synchronous motor.

For studies of the localization of the potential changes, the microelectrode solution was replaced by 10% $K_3Fe(CN)_6$ in 1 M KCl, and the electrode was connected to the oscilloscope for recording. The mercury drive was set in motion and allowed to operate until the first deflection occurred on the oscilloscope screen; the oscilloscope was operated with an internally triggered sweep at a speed of 3 cm per second. As soon as the first deflection occurred, the mercury flow was stopped and a 6-volt current was passed through the electrode for 90 seconds. A drop of 2% $FeCl_2$ was then added to the solution in the immediate vicinity of the electrode, and a deposit of Turnbull's blue was formed by reaction of the ferrous ion with ferricyanide drawn from the electrode tip by the 6-volt current (Bultitude, '58). The electrode was then withdrawn and the skin was fixed in neutral formalin, dehydrated, embedded in Tissuemat, sectioned at 10 μ, and stained with eosin.

RESULTS

Skins were penetrated either from the epidermal or dermal side in separate experiments. In every case, the potential difference measured before penetration was a typical resting potential of 20 to 50 mv. As the electrode moved through the skin, the potential difference decreased in two discrete steps, the final value being that of the asymmetry potential between the two electrodes, which was always less than 10 mv. Figure 2 is a tracing of a typical recording potentiometer record of a penetration from the dermal (inner) surface. The vertical scale represents the distance traveled by the electrode tip, as calculated from the rate of chart movement and the velocity of the tip. Figure 3 is a similar tracing of a penetration from the epidermal surface. Figure 4 is an oscilloscope trace of potential changes during penetration from the dermal side; no attempt was made at calibration of voltage or distance in this experiment, which was typical of the sorts of records obtained in dye-marking experiments. In such experiments, however, the movement of the electrode was stopped at the first potential change.

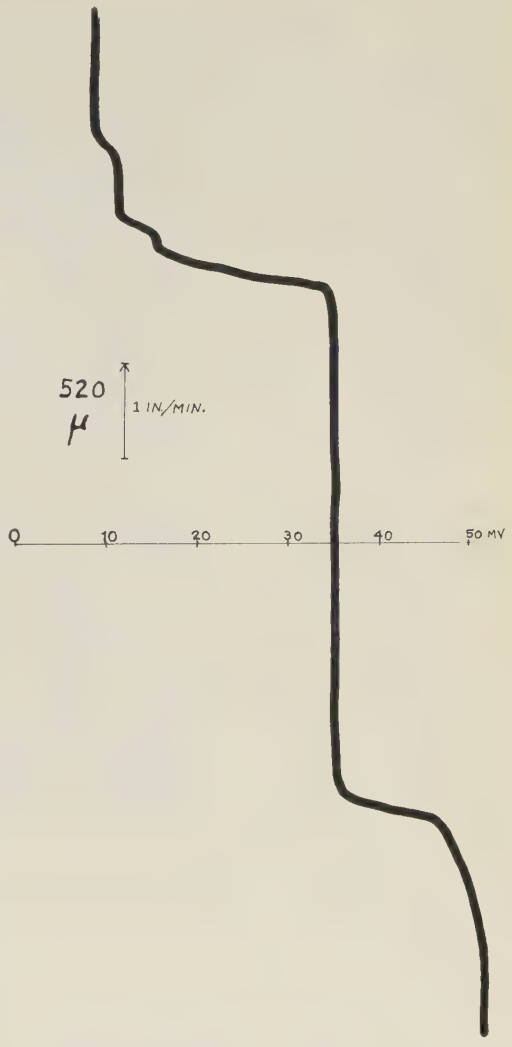

Fig. 2 Tracing of a potentiometer record of potential changes during penetration of frog skin from the dermal surface by a glass microelectrode filled with KCl. Abscissa: Potential, millivolts. Vertical scale: distance of electrode travel.

The thickness of the fresh skin used in experiments such as those represented in figures 2 and 3 was estimated by placing the skin between parallel metal plates and measuring the thickness of skin plus plates with a micrometer. The skin was then removed and the thickness of the plates alone was measured. The skins used ranged from 195 to 260 microns in thick-

ness. After fixation and preparation for microscopic examination, the range was 225–325 μ, with a mean ratio of thickness of fixed to that of fresh skin of 1.22. A fixed skin is shown in figure 5, with the approximate thickness of the major layers of a fresh skin indicated; these values are calculated on the assumption that all the layers swell in the same ratio during preparation. It is clear, in comparing the distance scale of figures 2 and 3 with figure 5, that the skin suffered considerable distortion during electrode penetration, since the total distance traveled by the electrode is considerably greater than the thickness of the skin.

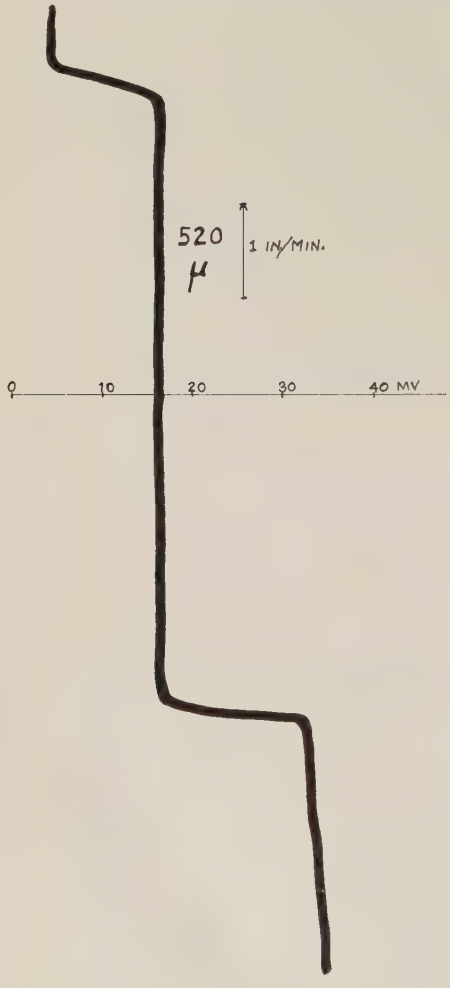

Fig. 3 Tracing of a potentiometer record during penetration of frog skin from the epidermal surface. Abscissa: millivolts. Vertical scale: Distance traveled by electrode.

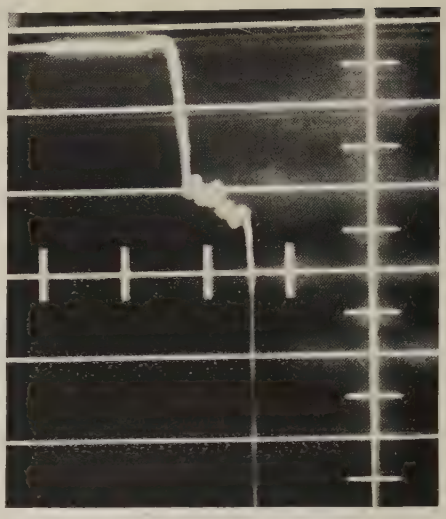

Fig. 4 Oscilloscope trace during penetration of frog skin by a glass microelectrode filled with KCl. Abscissa: time (0.3 sec.) Ordinate: potential (uncalibrated).

This distortion could be minimized by mounting the skin on an unperforated rubber stopper. Under these conditions, the system rapidly became polarized, and accurate potential measurements could not be obtained. However, the loci of the potential drops could be readily identified by sharp changes in slope in the potentiometer records. In one such record, the electrode traveled 650 μ in penetrating from the epidermal side; the skin thickness was 243 μ, a ratio of travel to thickness of 2.67. The first potential change occurred after 234 μ of travel, or if the same ratio holds, 87 μ below the surface of the epidermis. The second drop occurred when the electrode had traveled 416 μ beyond the site of the first drop, or through 156 μ of skin. By comparison with figure 5, it will be seen that the first drop might well have occurred in or near the *stratum germinativum*, but the second drop must have occurred well below this, and near the inner surface of the skin. Repeated studies of this type have always shown that, whichever the direction of penetration, the first drop always occurs close to the surface of the skin, and the second drop occurs close to the opposite surface; in any case, the distance from the surface to the

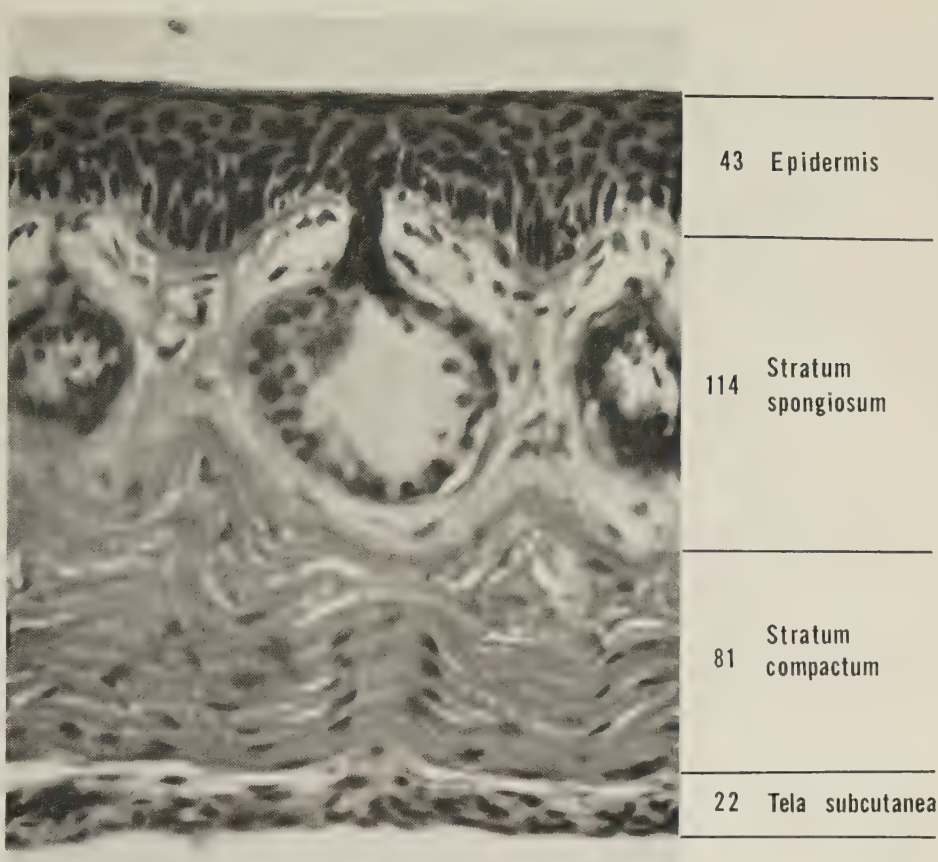

Fig. 5 Cross-section of normal frog skin to show principal layers. Approximate thickness of 260 μ. Actual thickness of this section after fixation, sectioning and staining 352.5 μ.

first drop is always much less than that from the first drop to the second.

In view of the distortion which the skin undergoes during penetration of the microelectrode, and the swelling which occurs during preparation for microscopic examination, both of which may be different in the different skin layers, the dye-marking technique appears to offer a more reliable means of localizing the potential drops. Figure 6 is a section of skin marked with Turnbull's blue at the first potential drop when the skin was penetrated from the epidermal side. The principal concentration of dye is evidently in the region of the *stratum germinativum*, though some dye has spread laterally below the dermo-epidermal junction, and some has diffused through the *stratum spongiosum* of the dermis. Figures 7 and 8 are sections of skins marked at the site of the first potential drop when penetration was from the dermal side. The spot is clearly localized in the *stratum compactum* just inside the *tela subcutanea*. Marking experiments of both types have invariably given the same results. Attempts to mark the second drop by this technique have never resulted in formation of a definite dye spot. The sections in such experiments generally show an electrode scar penetrating the entire thickness of the skin.

DISCUSSION

The results reported here are in agreement with those of Engbaek and Hoshiko ('57) in demonstrating that the potential difference across the isolated frog skin is the sum of two spatially separated potential differences with the same sign. We

are also agreed that the first difference is located in the general region of the epidermis. Engbaek and Hoshiko ('57), however, conclude that ". . . the second jump may correspond to the junction between epidermis and corium." Our results are in complete disagreement with this conclusion, and point to the *tela subcutanea* as the site of the second potential difference. Engbaek and Hoshiko ('57) used a hand-operated micrometer screw to drive their electrodes, and moved the electrode in discrete steps, with a measurement at each step. Their published records show great irregularities, so that it is difficult to decide which of the variations in potential represent the "jumps." They consider that the second jump occurred 57 to 166 μ below the epidermal surface, and report 4 cases out of 15 in which a third drop occurred at 140 to 170 μ. In more than 50 penetrations, we have never observed a third drop of any magnitude comparable to that of the two main drops. They made no penetrations from the dermal side, and offer no estimate of the degree of distortion involved in their measurements. Their major conclusions are based on coincidence of frequency distributions between electrode positions at the time of the first and second drops, and the thickness of the epidermis estimated in fresh skins. The errors inherent in their measurements—and in ours using similar methods—appear to be sufficient that no decision between their conclusions and ours can be made on the basis of these measurements alone. The same argument cannot be applied to the dye-marking experiments, which appear to us to be conclusive in localizing the second drop in the region of the *tela subcutanea*. In earlier studies, Ottosen et al. ('53) had concluded that the entire potential difference is localized at a single membrane. They penetrated the skin from both sides, but removed the *tela subcutanea* and *stratum compactum* before the experiment; their results are then surprising only in that they were able to measure any potential difference at all.

Fig. 6 Cross-section of frog skin marked after penetration from epidermal surface to site of first potential change.

The question of the exact locus of the potential drop is of importance in attempts to understand the mechanism of ion transport and skin potential. Linderholm ('54) developed a theory of sodium transport based on the assumption that these phenomena involve a single membrane, and Ussing ('48, '52, '54, '59) has offered several explanations of the relation of sodium transport and potential based on the assumption that the phenomena involved are localized in the *stratum germinativum*. From our results, and the theoretical demonstration (Scheer, '60) that the active transport process contributes to the electromotive force of the system in which transport occurs, it seems that we may localize the sodium pump in frog skin at two sites, one in the epidermis and the other in the *tela subcutanea*. This localization either indicates that there are two pumps in tandem, or that the pump has two parts spatially separated.

SUMMARY

1. The potential difference across isolated frog skin is the sum of two discrete potential differences of the same sign.

2. When the skin is penetrated by a microelectrode, the first potential change occurs when the electrode has moved only a short distance below the surface of the

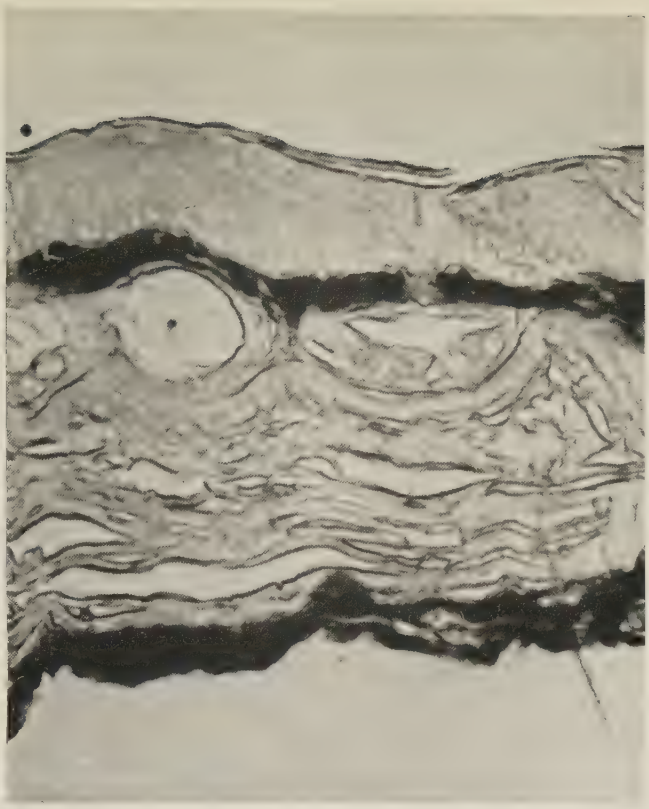

Fig. 7 Cross-section of frog skin marked after penetration from the dermal side to the site of the first potential change. The dye spot is seen as a small dark mark just above the tela subcutanea in the center of the figure.

Fig. 8 Cross-section of frog skin marked after penetration from the dermal side to the site of the first potential change. The dye spot is seen in the stratum compactum.

skin, whether the penetration is from the dermal or the epidermal side.

3. The distance between the sites of the first and the second potential changes is always greater than the distance between the skin surface and the site of the first change.

4. When the electrode is stopped at the site of the first change, and its position marked, the tip appears to be just inside the epidermis when penetration is from the epidermal side, and just inside the *tela subcutanea* when the penetration is from the dermal side.

5. From these observations we conclude that the loci of the electromotive forces in frog skin are the *stratum germinativum* of the epidermis and the *tela subcutanea* on the inner surface of the dermis.

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Non-Osmotic Water Movement Across the Isolated Frog Skin[1]

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In recent years the subject of active water transport has received considerable attention. It is easy to show in a variety of prepartions, both cellular and epithelial, that net water movement occurs between bathing solutions of the same osmotic pressure. This was shown for the frog skin first by Reid (1892). Later a detailed study was made by Huf and published in a series of papers (e.g., '36). An extensive literature on the mammalian intestinal mucosa has been reviewed recently (Durbin et al., '58). The case of active extrusion of water from mammalian cells (as distinct from translocation across epithelia) has been argued by Robinson ('54). In every case the net flux of water requires an input of metabolic energy; poisoning metabolism results in cessation of water movement. It would appear that the evidence for active water transport is so strong that its existence would be unquestioned.

Such acceptance has, however, not been forthcoming, and the reasons are easy to appreciate. In virtually every system across which water movement occurs there is also an active transport of ions—sodium in the frog skin, mammalian intestine and renal tubule, hydrogen and chloride in the gastric mucosa, and so on. The criteria for delimiting active transport of ions are reasonably clear-cut (Ussing, '49; Scheer, '58), but criteria for active water movement are not simple, particularly when the preparation is also transporting salt in the same direction. The reason is that the movement of water may be coupled with the ionic fluxes (the mode of such coupling need not be developed in detail here, but tight osmotic linking and electro-osmosis are two possibilities). In such an event the apparently simple tests for active water transport fail; no gross osmotic asymmetry need exist between the two solutions bathing an epithelium, and the requirement for a metabolic input may represent the energetic demands of the ion transporting system. It should be stressed that coexistence of ion and water movement does not *require* that the latter be linked with the former. However, where the possibility of linkage exists the burden is certainly on proving that they are independent.

Actually there have been few really careful attempts to separate the two fluxes. Huf et al. ('57) showed that there was good correlation between inhibition (and stimulation) of salt movement and changes in net water flux across the *in vitro* frog skin. And, in the intact toad, Kalman and Ussing ('55) observed that abolition of sodium transport (by replacing the sodium in the external bathing solution with isotonic sucrose) resulted in cessation of water uptake through the skin. On the other hand, it was also noted that there was little correlation between intensity of sodium transport and water influx; the water uptake increased in the longer experiments, while the salt uptake decreased. Careful study of the relative rates of absorption of sodium, chloride and water through the rat ileum (*in vivo*) showed that water absorption was directly proportional to the net solute flux. Water absorption halted precisely where the net uptake of solute stopped (Curran and Solomon, '57). The elegant experimental work seems to show unequivocally a tight, obligatory linkage between ion and water transport in this preparation. Although

[1] This investigation was supported by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171, and by research grant G-4925 from the National Science Foundation.

we will not be concerned with water movement from cells it is worth noting that here too, the interpretation of data purporting to support the case for active extrusion has been severely criticized (Leaf, '56).

Since most of the data for the frog skin has been gathered in the course of work designed for another purpose it was decided to reexamine the question of linkage of sodium and water movement. This preparation has been studied in detail, and techniques have become available for attempting to separate the fluxes. As noted, metabolic inhibitors are not very useful for this purpose, but a variety of treatments have been described which will cause a change in sodium movement with no important effect on metabolism. The purpose of this paper is to report some of the results obtained in such an investigation.

METHODS

Two preparations were used. The calf skin bag described by Maxwell ('13) was employed extensively, in part because of the ease of preparing and running several preparations simultaneously, and in part because paired preparations could be used in some experiments in which one member of the pair served as a control while the other was subjected to experimental treatment. The bags were filled and immersed in a large volume of well-aerated solution of the desired composition. After an hour for equilibration they were removed, and excess moisture was removed by dragging them across the lip of a dry beaker. Blotting on filter paper was found to cause changes in water permeability in a few experiments, and hence was not used. The bags were then weighed in a covered weighing bottle. Precision of the method was $\pm$ 3.3 mg (20 successive weighings). For 12 cm^2 of skin moving water at the rate of 2 $\mu l/cm^2$/hour the uncertainty in a series of two-hour measurements is thus about $\pm$ 7%, although a single weighing might deviate more than this.

In some experiments each skin served as its own control. In these cases at least two, and sometimes three two-hour measurements were made serially before the introduction of an experimental variable. There was often a tendency for the rate of water movement to increase for several hours (the same phenomenon was noted by Kalman and Ussing in intact toads), and this limits the usefulness of the method. In addition, such experiments are very long (seldom less than 12 hours), although it was easy to show that the preparation was still viable at the end of the period (both sodium and water continued to move, and the movement could be stopped with an inhibitor). In many of the experiments bags from the same animal were paired, one serving as a control throughout, the other as the experimental preparation.

There were several disadvantages in using this method apart from the duration of the runs. Serial measurement of sodium fluxes was difficult due to the low specific activity of the isotope available here (Na^{22}), and addition of test compounds could be made to one side of the preparation only. Hence some of the experiments were conducted on sheets of ventral abdominal skin essentially as described by Koefoed-Johnsen and Ussing ('53). Changes in volume of the solution in one of the chambers was measured with a precision bore microburette. The precision was about the same as with the skin bags. Volume measurements were made hourly, usually in duplicate. Either potential difference or short circuit current was monitored. Both electrical parameters serve as an index of sodium transport. Substantially the same results obtained here as in the work with bags prepared from the legs.

Isotonic, sodium-free solutions were prepared with either choline chloride (Kirschner, '55), magnesium chloride (Zerahn, '55) or sucrose. In the case of choline Ringer's the concentrations of all constitutents except sodium was the same as for normal Ringer's solution. Magnesium Ringer's had a higher chloride concentration and no sodium, but was otherwise similar to normal Ringer's. Isotonic sucrose solution was made to contain the same concentrations of potassium and calcium (chlorides) as in normal Ringer's solution. All solutions were buffered with "tris" at pH 8. Periodic measurement of freezing point depressions showed that the total solute concentrations were within 10% of the estimated values. Such a

check is important in the case of choline which has a tendency to decompose even on storage at low temperature (choline purified by extraction with isopropyl alcohol and ether does not have characteristic "fishy" odor of a tertiary amine. But the odor will develop on storage).

Water movement in the absence of ion transport

The first experiments involved replacing sodium in the external solution with a sodium-free, isotonic saline. Table 1 shows the result of two representative experiments of this type. Skin bags prepared from two animals were filled with 1.5 ml of normal Ringer's. One of these bags was placed in a beaker containing 100 ml of choline Ringer's solution; the other preparation from this animal went into normal Ringer's. One bag from the second animal was placed in 100 ml of magnesium Ringer's; the other was placed in normal Ringer's. After an hour for equilibration each bag was weighed. Weighings were then made every two hours. At the end of the experiment the fluid in the bags was collected for sodium analysis (these data are not shown), the bags were opened, slit lengthwise, and unrolled on a sheet of graph paper. The outline of the bag was traced and used for measurement of the area of the skin. The fluxes (microliters per cm² per hour) are shown in the table. The water movement in bag C2 was essentially the same as that across bag E2. In this pair, then, replacing sodium by magnesium had no effect on water uptake. The situation for the other pair differed quantitatively in that the net flux from choline Ringer's was only about one-third that across the control. Such a difference was not invariable, but it was noted often. In a series of 27 two-hour periods involving 9 such pairs the average net water fluxes were: for the controls (normal Ringer's outside) 1.6 μl/cm²/hr., and for the experimental bags (choline outside) 0.8 μl/cm²/hr. Cumulative data for magnesium show that water influx is not appreciably different from the controls. Thus for 43 two-hour periods in 14 paired preparations the controls moved 1.9 μl/cm²/hr., while 2.0 μl/cm²/hr. moved across from magnesium Ringer's. The reason for this difference between choline and magnesium is not known. However, the important point established by these data is that a net flux of water can occur in the absence of solute transport as well as with no osmotic gradient. For magnesium, at least, the magnitude of this non osmotic flow is about the same as that across skins engaged in sodium transport.

This phenomenon is certainly at variance with previous reports that the use of a sodium-free external solution abolishes water movement. In the work reported in the past the sodium-free solution has been made isotonic with sucrose (Koefoed-Johnson and Ussing, '53; Kalman and Ussing, '55). This type of experiment has been repeated, and the data in table 2 show that water influx not only stops under these conditions, but that a small net *outflux* occurs. This has been noted in every preparation run, both with sucrose and glucose solutions; it was apparent, too, in the data published by Kalman and Ussing on intact toads.

While we have no certain explanation for the difference betwen skins bathed outside by electrolytes and those bathed by non electrolytes, this phenomenon suggests

TABLE 1

Water influx across paired frog skins: choline and magnesium Ringer's

Skin	Non-penetrating ion[1]	External sodium	Net water influx		
			0–2 hrs.	2–4 hrs.	4–6 hrs.
		microeq./ml	*microliters/cm²/hr.*		
C1	—	106	3.6	3.6	5.3
E1	Choline	—	1.1	0.9	1.7
C2	—	106	0.6	0.7	2.7
E2	Magnesium	—	0.8	0.8	1.4

[1] Choline and magnesium Ringer's were isotonic with the sodium Ringer's bathing the inside of the skins.

TABLE 2

Water influx across paired frog skins: sucrose Ringer's

Skin	Na out[1]	Net water influx[2]		
		0–2 hrs.	2–4 hrs.	4–6 hrs.
	microeq./ml	*microliters/cm²/hr.*		
C1	109	1.9	3.3	3.9
E1	0	−0.8	−0.5	−0.3

[1] Skin E1 bathed outside by sucrose Ringer's.
[2] Positive values denote a net influx of water; negative values a net outflux.

TABLE 3

Water influx across frog skins: sucrose Ringer's plus sodium

Skin	Net water influx (microliters/cm²/hr.)				
	Sucrose Ringer's		Sodium Ringer's : Sucrose Ringer's (15:85)		
	0–2 hrs.	2–4 hrs.	4–6 hrs.	6–8.8 hrs.	8.8–10.8 hrs.
E1	−0.3	−1.4	−0.4	−0.4	−0.3
E2	−0.7	−0.5	−0.4	−0.6	−0.2

yet another test for linkage between salt movement and water flux. It is easy to show that if sodium is added to the sucrose solution an active sodium transport will occur as usual. If coupling is obligatory initiation of ion movement should reverse the direction of water movement. Two such experiments are shown in table 3. The bags (from two animals) were set up with isotonic sucrose bathing the outside and measurements for two control periods showed the usual small outflux. At this time both bags were transferred to a solution in which 15% of the sucrose had been replaced with normal Ringer's solution (Na concentration about 17 mM/l). Essentially no change occurred in either the direction or magnitude of the water flow. Sometimes the rate of water efflux did decrease slightly, but even when noted the change was small. In no case was an influx of water generated. The sodium influx in these experiments was usually between 0.1 and 0.2 μM/cm²/hr. The same kind of results have been obtained with toad skins.

Another test for linkage involves inhibition of sodium movement by some method which does not interfere with energy metabolism. One such method entails the use of cholinesterase inhibitors (Kirschner, '53). In such an experiment it is desirable to monitor sodium movement in order to ascertain the effectiveness of the inhibitors. For this reason the ventral abdominal skin was used. It was bathed on both sides with normal Ringer's solution, and the preparation was short circuited. Current was used to compute the net sodium influx, and measurements of water movement were made hourly. After three control periods eserine was added to both chambers (5×10^{-3} M). The drug is active only on the inside of the skin, but addition to both sides prevented any osmotic asym-

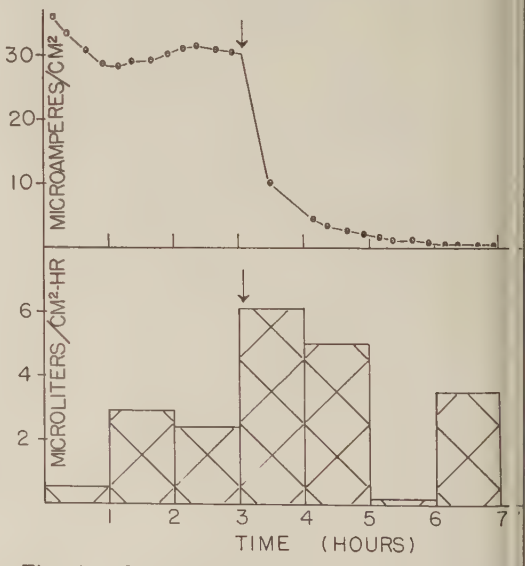

Fig. 1 The effect of eserine on net sodium transport and water influx. The skin was equilibrated for two hours before any readings were taken. Eserine sulfate (5×10^{-3} M) was added to both chambers at the arrow.

metry from developing (fig. 1). It can be seen that although ion movement was completely inhibited the movement of water continued for the duration of the experiments. Eserine also had little effect on water movement across bags of calf skin, although in these preparations salt fluxes were not measured.

Water movement linked with sodium transport

The experiments already described show that a net movement of water can occur in the absence of simultaneous sodium transport. There is, however, much evidence that water movement may, under some conditions, be coupled with salt movement. Occasionally a skin bathed on the outside with an inert Ringer's solution shows a very low water movement. With such a preparation it is sometimes possible to initiate an influx of water by adding sodium to the outside solution; presumably the generation of a net inward sodium flux causes the water movement. A much more reproducible change follows the addition of atropine to the solution bathing the outside of the skin. This drug causes a sharp increase in sodium transport (Kirschner, '53). A pair of bags were set up with normal Ringer's on both sides. Weighings for this experiment were made each hour. At the end of 4 hours atropine sulfate (5×10^{-3} M) was added to the outside of bag E1. The other bag was left in normal Ringer's without atropine. Table 4 shows that a sharp rise occurred in water movement across the atropine treated skin with the result that the flow is very high for the next three hours. A smaller rise occurred in the control; as noted earlier, such a change is frequently seen in long experiments.

DISCUSSION

Although coupling of water movement and sodium transport may occur in the frog skin, these experiments show that the former will occur in the absence of solute transport. The data described in table 1 for a skin exposed to choline Ringer's have also been obtained by J. Maetz.[2] He has also found that the action of oxytocin on water movement often differs completely from its action of sodium transport, and this too, seems to indicate that these fluxes are independent of each other. Since no external osmotic gradients are necessary, and since the water influx is dependent on metabolic energy, the requisites for active transport seem to be satisfied. There are reasons, however, for hesitating before accepting this conclusion.

For one thing, the experiments on replacement of sodium simply do not make a consistent picture. The quantitative difference between the fluxes from magnesium and choline may be unimportant. It may be the result of depression in activity of the skins by impurities in the choline. The behavior of the skin in the presence of an isotonic non electrolyte is more perplexing, and no explanation can be offered for the cessation of flow under these conditions. The small outward movement may have been the result of dehydration of the skin itself, although the evidence for this is tenuous. The presence of a large excess of sucrose or glucose does not impair the ability of the skins to transport sodium; in fact, skin bags bathed outside by 25 mM NaCl in isotonic sucrose are used routinely here for classroom demonstration of active

[2] I wish to thank Dr. Maetz for permission to quote his still unpublished work. An abstract of this work, which will be presented at the First International Endocrinological Congress, is in press.

TABLE 4

Effect of atropine on water influx

Skin	Na out	Net water influx (microliters/cm²/hr.)						
		Control				Atropine		
		1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	7 hr.
	mM/l							
C1	114	1.1	1.1	1.0	1.1	2.4	2.0	2.0
E1	114	1.1	1.3	1.8	1.7	4.4	5.0	5.1

sodium transport. Yet water influx fails completely under these conditions.

It is not fashionable to bring teleological arguments into a discussion of mechanism, but one of the reasons for application of caution in accepting this as a case of active transport has just such a base. It would seem to make little functional "sense" for the frog, a semiaquatic, freshwater animal, to expend energy in moving water into the body fluids. The normal osmotic entry places the animal in a situation in which energy must be expended to eliminate water.

Several alternative explanations have been considered. The skins themselves contain appreciable quantities of ions (Huf et al., '57), and if they diffused out into the solution bathing the inside some water might accompany them. For example a freshly excised skin contains about 0.25 μeq. Na/mg dry weight. An average skin bag weighs about 100 mg dry and hence contains about 25 μeq. Na. If half of this diffused into the inside solution, it could cause the movement of about 100 μl of water, assuming a tight osmotic linkage. This would generate a flux of about 1–2 μl/cm²/hr. over an experiment of 6–10 hours. However, measurements on many preparations showed that there is no loss of Na, K, Ca, or phosphate from the skin during the course of an experiment. The values for each of these after prolonged exposure to one of the inert external solutions is essentially the same as the corresponding value from a piece of freshly excised skin. Another possibility, one frequently mentioned in the literature, is that the water movement is caused by electro-osmosis. The fact that the flux occurs only when an electrolyte bathes the outer surface seems to indicate that some electrical characteristic of the skin in involved. However, the following observation would seem to argue against this mechanism. When the skin is bathed by choline or magnesium there is a small potential difference across it, usually 3–10 mv outside positive. When the skin is bathed on both sides by normal Ringer's, and sodium transport is inhibited by eserine there is a small residual potential of about the same magnitude, but with the opposite polarity (i.e., 1–3 mv, inside positive). In both cases the direction of water movement is inward, and the magnitudes are approximately the same. However, the possibility that electro-osmosis is involved is being further investigated.

Whatever the mechanism underlying the non osmotic movement of water across the isolated frog skin, it is apparent from the data in this paper that it is not necessarily a concommitent of the active transport of sodium.

SUMMARY

A study of the net transfer of water across isolated skins of *R. pipiens* yielded the following information. A net water influx occurred when the outside of the skin was exposed to an isotonic solution of a non pentrating electrolyte. The net transfer was greater when magnesium was used as the inert electrolyte than when choline was used, but a measurable influx occurred from isotonic solutions of both. When skins were bathed outside by normal (sodium) Ringer's solution water influx was little changed even when sodium transport was inhibited by eserine. When the external solution was non ionic water influx stopped and a very small efflux was noted.

Evidence was also presented to show that water movement might, under some conditions, be linked with salt transport.

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The Effects of Hydrocortisone on Glycogen Storage and Phosphorylase Activity of Frog Liver

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The results of many studies have shown that cortisone causes a variety of effects in animals. Some of these might be due to: (1) the organ or material being investigated and (2) physiological condition of the investigated animal. The first is suggested by the works of Trémolières and his coworker ('56). For example, they demonstrated that male rats injected with cortisone showed increments in liver and kidney weights but decrements in adrenal weights (Trémolières and Ségal, '56). Part of the increased liver weight was shown earlier by one of these investigators to be due to glycogen storage, and the other to protein synthesis (Trémolières, Derache and Griffaton, '54). Goodlad and Munro ('59) believed that cortisone acted as a catabolic agent in the rat body and released amino acids that were used in liver protein synthesis. Cannon, Frazier and Hughes ('56) had previously recognized the existent opinion that cortisone acted as a catabolic agent. Cortisone was shown by Grewe and Williams ('58) to raise the glycogen content of certain skeletal muscles of mice. In this same category is the effect of cortisone on phosphatase activity from different sources. Souilairac, Souilairac and Teysseyre ('55) demonstrated that cortisone elevated rat muscle phosphatase activity but lowered that from the ect chorion. That the physiological condition of the animal might be important in the response of animals to cortisone is illustrated by the works of Laron and his coworkers ('56, '58). It was found that cortisone lowered the serum phosphatase level in rachitic rats (Laron, Canalas, Jr. and Crawford, '56). The hormone might cause an opposite effect in normal rats since Laron, Muhlethaler and Klein ('58) found that the serum phosphatase level was raised in four animals and lowered in two others.

Most physiological studies concerning the effects of cortisone have (1) utilized mammals as the experimental material, and (2) employed the technique of daily rather than single dosage injections. It might be of some interest (1) to determine whether the same effects of the hormone known to occur in mammals are also manifested in another vertebrate, such as the frog, and (2) to determine whether single dosages have the same physiological effects as multiple dosages. Therefore the present study will show (1) that cortisone has the same effect on glycogen storage in the frog liver in short-term experiments as has been demonstrated for rat liver; (2) that the phosphorylase level of frog liver is elevated following the injection of cortisone as is known to occur for rat liver; and (3) that the pattern of increase in glycogen storage and in the level of phosphorylase activity of frog liver is similar to that demonstrated for the liver of the rat.

MATERIALS AND METHODS

Hydrocortisone (from Merck Company) was made into three separate solutions, of 1.0 mg%, of 5.0 mg%, and 10.0 mg%.

The frogs (*Rana pipiens*) were divided into eight groups, each of which contained from 10 to 20 animals. Two groups were used as controls, the others as the experimentals. One control group was used to determine the mean glycogen content of the liver, the other to determine the mean liver phosphorylase activity. All experimental animals were injected in the pleuroperitoneal cavity with 0.5 ml of hydrocortisone. Two groups were injected with 1.0 mg%, two groups with 5.0 mg%, and two groups with 10.0 mg%. After injection, one group of each pair was used for the glycogen determinations, and the other for phosphorylase activity. All ani-

mals were sacrificed 48 to 56 hours following injection.

Each frog was pithed, weighed, the liver quickly excised and freed of adhering tissues and weighed. For the glycogen determinations, the livers were homogenized in a chilled Waring blendor containing 15.0 ml of cold 10% trichloroacetic acid (TCA). Glycogen was extracted from the homogenate according to the method of Good, Kramer and Somogyi ('33) as outlined by Umbreit, Burris and Stauffer ('59). The reducing sugar that was formed upon hydrolysis of the glycogen was measured by the method of Folin and Wu ('20) in a Coleman Junior Model A spectrophotometer at 420 mμ in 12 × 75 mm cuvettes. The milligrams of reducing sugar was converted into anhydrous glycogen by using the conversion factor (0.927) of Umbreit, Burris and Stauffer ('59). The results were expressed in terms of mean milligrams of glycogen that the livers contained and as the mean milligrams of glycogen/milligrams wet weight of liver tissue.

For the phosphorylase determinations, animals were pithed, weighed and the livers excised as for the glycogen determinations, but the organs were homogenized in a chilled Waring blendor containing 40.0 ml of cold Ringer's solution without glucose. The homogenate was filtered through Whatman no. 40 filter paper and the filtrate was used for the enzyme determinations according to the method of Sutherland ('55) as outlined by Colowick and Kapalan ('55). The reaction mixture was taken at zero time and incubated in a constant temperature bath for 30 minutes at 30°C. At the end of the incubation period, the reaction was stopped by adding three volumes of 10% TCA to the mixture. The precipitated proteins were separated from the solution by filtration through Whatman no. 40 filter paper. The filtrate was used to determine the inorganic phosphate liberated by the enzyme and to determine the nonprotein nitrogen (NPN) content of the sample. The inorganic phosphate was determined by the method of Fiske and Subbarow ('25) as published by Hawk, Oser and Summerson ('47). The density of the solutions was determined in the Coleman Junior Spectrophotometer at 660 mμ in 12 × 75 mm cuvettes. Phosphorylase activity of the liver was calculated on the basis of the micrograms of inorganic phosphate liberated from the substrate by the 1.0 ml crude enzyme sample/micrograms NPN/30 min at 30°C.

The micro-Kjeldahl method of Folin and Wu ('19) as described by Hawk, Oser, and Summerson ('47) was used to determine the NPN of the sample. The density of solutions was determined in a Coleman Junior Spectrophotometer at 480 mμ in 12 × 75 mm cuvettes. The results were expressed in terms of the mean micrograms of NPN/milligram of liver wet weight.

All data were obtained from duplicate determinations. The tabulated data include the mean (M), the standard error of the mean (S.E.) (table 1 only), the standard deviation of the mean (S.D.) and the probability (P) values. A probability of less than 0.05 was considered significant in accordance with the suggestion of Fisher ('50).

RESULTS

Tremendous variations existed in the mean body and liver weights of animals within the same group. Similar variations occurred in individuals when the weight of the body was compared with the weight of the liver. For example, some small animals had comparatively medium or large livers, whereas others had small livers. The same conditions existed for the large frogs.

The results of the experiments for determining the liver glycogen content are summarized in table 1. The table shows that the descending order of glycogen concentration in the livers of the 4 groups of animals was 1.0 mg% injected, controls, 10.0 mg% injected, and 5.0 mg% injected. The smallest livers were from animals injected with 5.0 and 10.0 mg% hydrocortisone. A difference of 10.0 mg occurred between their liver weights, and 0.11 mg between their glycogen contents. The largest livers were from the controls. They weighed approximately 270 mg more than the largest livers from injected animals but contained approximately 1.3 mg less glycogen.

TABLE 1

Glycogen content of frog liver following injection of Hydrocortisone[1]

Animal	Body weight (gm)				Liver weight (mg)				Glycogen content of liver (mg)				Mg glycogen/mg liver wet weight			
	M	S.E.	S.D.	P	M	S.E.	S.D.	P	M	S.E.	S.D.	P	M	S.E.	S.D.	P
Control	38.93	1.54	6.62	0.01	1340	96.90	433.18	0.01	15.63	0.33	1.39	0.01	0.012	0.075	0.323	0.01
1.0 mg % hydrocortisone injected (0.5 ml)	29.42	1.55	6.66	0.01	1070	93.15	417.00	0.01	16.32	0.37	1.61	0.01	0.016	0.090	0.385	0.01
5.0 mg % hydrocortisone injected (0.5 ml)	37.53	1.58	6.79	0.01	940	142.19	635.48	0.01	14.38	0.03	0.72	0.01	0.016	0.070	0.343	0.01
10.0 mg % hydrocortisone injected (0.5 ml)	34.07	1.45	6.23	0.01	950	49.45	221.02	0.01	14.49	0.40	1.72	0.01	0.015	0.052	0.227	00.1

[1] M, mean; S.E., standard error of the mean value; S.D., standard deviation of the mean value; P, probability.

TABLE 2

Phosphorylase activity of frog liver following injection of hydrocortisone[1]

Animal	Body weight (gm)			Liver weight (mg)			Mg phosphate liberated/1.0 ml enzyme/30 min.			μg NPN contained in liver			μg phosphate liberated/30 min./ μg NPN		
	M	S.D.	P	M	S.D.	P	M	S.D.	P	M	S.D.	P	M	S.D.	P
Control	29.97	5.11	0.01	851.0	278.0	0.02	35.00	9.0	0.01	301.0	53.0	0.01	0.117	0.031	0.02
1.0 mg % hydrocortisone injected (0.5 ml)	28.79	3.80	0.05	789.3	194.22	0.05	29.40	6.33	0.02	223.9	46.91	0.05	0.131	0.011	0.01
5.0 mg % hydrocortisone injected (0.5 ml)	29.63	7.60	0.02	720.0	250.04	0.05	26.10	7.80	0.05	206.4	55.89	0.05	0.127	0.009	0.02
10.0 mg % hydrocortisone injected (0.5 ml)	23.98	3.63	0.05	656.0	173.91	0.05	25.80	7.47	0.05	190.6	25.50	0.05	0.135	0.016	0.05

[1] M, mean; S.D., standard deviation of the mean value; P, probability.

The differences between the glycogen content of the control and experimental groups were more apparent when the glycogen content was calculated on the basis of the quantity of liver (weight) that produced the milligrams of glycogen. As seen in table 1, the controls contained approximately 0.004 mg of glycogen less per milligram of wet weight of liver than those of the experimentals. The table shows further that livers of each experimental group contained approximately the same quantity of glycogen. Since all of the data were below the 5% level of confidence, they were considered significant.

The results of the experiments on determining the phosphorylase activity of the livers are summarized in table 2. As shown in the table, the control livers weighed approximately 70–200 mg more than those from the other groups and their phosphorylase activity produced from 5.0 to 10.0 μg more inorganic phosphate than the other animals. The lowest quantity of inorganic phosphate was formed by the enzyme from the animals injected with 10.0 mg% hydrocortisone. This latter group also had smaller livers than any other group. The decreasing order of inorganic phosphate yielded by the enzyme from the livers of different groups was the controls, 1.0 mg% hydrocortisone injected, 5.0 mg% hydrocortisone injected, and 10.0 mg% hydrocortisone injected. The same decreasing order existed for the liver weights of the animals.

As shown in table 2, a direct relationship existed between the size of the liver and the nonprotein nitrogen that the livers contained. The highest NPN value was obtained for the controls and the lowest was for the animals injected with 10.0 mg% hydrocortisone. The difference between these values was approximately 110 micrograms.

Table 2 shows further that the controls presented the lowest values when the inorganic phosphate liberated by the enzyme was compared with the NPN value. The highest phosphate value per unit NPN was for the animals injected with 10.0 mg% hydrocortisone. The difference between the lowest and highest values was 0.018 μg. The values of the experimental groups differed from each other by 0.004 μg.

All data presented in table 2 were less than the 5% level of confidence, hence were considered to be significant.

DISCUSSION

The results of these experiments suggest that the size of the animal is independent of the size of its respective liver, and the corresponding glycogen content of the structure. Some livers weighing 15.0 to 20.0 mg more than smaller ones yielded considerably less glycogen than the latter structures.

If one compares the average total glycogen content of the livers of the experimental with that from the controls, it could appear as if hydrocortisone has little or no effect on liver glycogen storage in short-term experiments. Such inference could be drawn from the fact that livers from two of the experimental groups (e.g., animals injected with 5.0 mg and 10.0 mg% hydrocortisone) yielded an average of approximately 1.0 mg less glycogen than the controls. However, if one compares the glycogen content of the livers in terms of the wet weight of the liver for the respective groups, it becomes apparent that the experimental groups contained approximately 33⅓% more glycogen than the controls. These results are in partial agreement with that of Trémolières, Derache, and Griffaton ('54). These investigators found that the liver mass of rats increased in two to 5 days following the injection of cortisone and ⅓ of this was from glycogen.

The concentration of hydrocortisone that was injected into the frogs had no effect on the quantity of glycogen stored in the liver. Approximately the same values were obtained for animals injected with 1.0 and 5.0 mg% hydrocortisone when the amount of glycogen obtained from the livers was compared with their corresponding weights. When these latter values are compared with those for the liver from 10.0 mg% hydrocortisone injected animals, a difference of only 0.001 mg of glycogen occurs. It is reasonable to suppose that this very slight difference is of no importance and could have arisen from any number of possible causes. The near-identical results suggest that the critical point of hydrocortisone stimulation of liver glycogen storage in these animals is probably less than the lowest concentra-

tion that was injected. One would suspect that beyond the critical level, hydrocortisone probably would have no additional effect.

A direct relationship was found to exist between the liver weight and the inorganic phosphate liberated by the phosphorylase. A similar relationship existed between the liver weight and its nonprotein nitrogen content. The larger livers had more enzyme activity and more non-protein nitrogen. Because of this relationship and the wide variations in the sizes of the livers, it was necessary to compare the activity of the enzyme from the different groups in terms of the units of phosphate liberated by the same units of nonprotein content of the liver. When this was done, the values of the experimental groups were approximately the same. The difference of only 0.004 μg is an infinitesimal quantity and is probably an artifact.

When the experimental values from the phosphorylase determinations were averaged, they represented an increased yield of inorganic phosphate of approximately 12% over that of the controls. Derache, Trémolières, Graffton, and Lowy ('56) found that cortisone only slightly increased the activity of glucose-1-phosphatase of rat liver, while the glycogen level was greatly increased. It appears that the pattern of our results with frog liver is in agreement with that of the earlier investigators.

SUMMARY

1. The glycogen content and the phosphorylase activity of frog (*Rana pipiens*) liver were determined after 48 to 60 hours following single pleuroperitoneal injections of 0.5 ml of 1.0 mg, 5.0 mg, and 10.0 mg% hydrocortisone.

2. Hydrocortisone in each of the preceding concentrations appeared to cause approximately a 33⅓% increase in liver glycogen in the frogs.

3. The phosphorylase activity of frog liver appeared to be increased approximately 12% after injection of hydrocortisone.

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